Petroleum Hydrocarbon Utilization by Fungi Isolated From *Detarium* Senegalense (J. F Gmelin) Seeds

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Abstract: Petroleum hydrocarbon-degrading fungi were isolated from *Detarium senegalense* seeds. An assessment of the relative ability of each fungus to degrade petroleum crude oil, diesel, unspent and spent engine oils, kerosene and *Detarium senegalense* oil extract, on minimal salt broth, was done measuring change in optical density read on a spectrophotometer. Five fungi were isolated from diseased namely: *Aspergillus flavus, A.niger, Mucor, Rhizopus,* and *Talaromyces*. The fungi isolated were used in the experiment and it was evident that all the fungi were capable of biodegrading the petroleum oil, though at different rates. *Aspergillus niger* had the highest ability to degrade unspent engine oil and *Detarium senegalense* oil extract while *Rhizopus* had the highest ability to degrade kerosene and diesel, and *Talaromyces* had the highest ability to degrade spent engine oil. There was fluctuation in the growth pattern of the fungi in the petroleum oil medium. The implication of this result was discussed. [The Journal of American Science. 2007;3(1):69-76].

Keywords: Oilseed; Biodegradation; Fungi; Oil pollution; *Detarium senegalense*

Introduction

Petroleum like all fossil fuels primarily consists of a complex mixture of molecules called hydrocarbons. In large concentrations, the hydrocarbon molecules that make up crude oil and petroleum products are highly toxic to many organisms, including humans (Alexander, 1994). The dominance of petroleum products in the world economy creates the conditions for distributing large amounts of these toxins into populated areas and ecosystems around the globe (Ojumu, 2004).

The most rational way of decontamination of the environment loaded with petroleum derivatives is an application of methods based mainly on metabolic activity of micro organisms (Leahy and Colwell, 1990). Microbial degradation is the major mechanism for the elimination of spilled oil from the environment (Colwell and Walker, 1977; Ibe and Ibe, 1984; Atlas, 1995). The ability to actively decompose specified fractions of petroleum oil is expressed by many micro organisms (Bartha and Atlas, 1977). Yuan et al (Yuan, Wei and Chang, 2000) suggested introduction of mixed cultures of bacteria and fungi, especially that not all components of petroleum – derived hydrocarbon mixture are decomposed simultaneously. However, single cultures of fungi have been found to be better than mixed cultures (Okerentugba and Ezeronye, 2003) and more recently, 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 micro organisms isolated from a totally unrelated environment (Ojo, 2005). The source of fungi for the biodegradation of petroleum oil becomes the issue. In most previous researches, the source of fungi is either selected from crude oil polluted soils or water (their natural environments), rhizoplanes, phyloplanes, food or crops and root tubers. Some other fungal source for biodegradation of petroleum oil, are the experimental use of mycorrhizal fungi and wood rotting fungi in the biodegradation of petroleum. In this laboratory, attention is paid to source for fungi from oilseeds in the tropical forest of Nigeria. It has been shown that fungal infection of oily seeds lead to deterioration of oils extracted from the seeds by changing the free fatty acid content, peroxide and saponification values, and also reduces the quantity of oil present in the seeds (Kuku, 1979, Adekunle and Uma, 1996). These are as a result of the utilization of the hydrocarbons that make up the oils by the pathogenic fungi with the aid of the lipase enzyme they produce (Adekunle and Uma, 1996). These fungi have been shown to be capable of utilizing the hydrocarbons present in petroleum (Adekunle and Oluyode, 2000). There is no report on hydrocarbon-utilization of fungi isolated from *Detarium senega*lense seeds. Therefore, the aim of this study is to investigate and document the capability of fungal species isolated from diseased *Detarium senegalense* seed to utilize and degrade hydrocarbons in crude petroleum oil and other petroleum products such as diesel, kerosene, spent and unspent engine oil.

Materials And Methods

Collection of Samples

Detarium senegalense seeds used for this investigation were collected from the local Oyingbo market in the Lagos area of Nigeria. Both healthy and diseased seeds were collected after which the diseased seeds were separated from the healthy ones on the basis of the presence or absence of visible symptoms respectively. The oil seeds were sampled from the market three times in the first month and monthly for the next two months.

The petroleum crude oil was obtained from Escravos oil field of Chevron Texaco (Nig.) Limited in Warri, Delta State, Nigeria.

Isolation and Identification of Hydrocarbon – Utilizing Fungi

About forty diseased seeds of *Detarium senegalense* were broken into smaller pieces and thereafter, surface sterilized by leaving them in a mixture of common bleach (Sodium hypo chloride solution) and sterilized distilled water in ratio 3:2 for a minute, while stirring the seeds with sterilized forceps. Afterwards, the seeds were rinsed with three changes of sterilized distilled water and were then used for isolation. Four seeds of *Detarium senegalense* were evenly distributed with the use of flamed forceps on each of the six Petri dishes with previously prepared potato dextrose agar (PDA). Afterwards, the plates were incubated at a temperature of 28 – 31^{o}C for 48hours or more depending on the rate of growth. To obtain pure cultures of the fungal isolates, developing fungal cultures were aseptically subcultured into fresh PDA plates and incubated until the fungus begins to sporulate followed by subsequent sub culturing and incubation a number of times until pure cultures consisting of only one type of fungus respectively were obtained. A part of the pure culture was then aseptically transferred into sterile agar slants which had previously been prepared in 14ml McCartney bottles. The bottles are then incubated till full growth of the fungus is observed and they then serve as stock cultures.

To identify the fungi, firstly, morphological studies, that is, examination of the size, shape, colour, spore formation and the number of days taken for the fungus to reach maximum diameter (9cm) of the Petri dish and the texture of fungal growth were observed. After 2 – 4 days of growth of the fungi, the spore – bearing mycelia were then carefully sectioned, teased out and stained on a slide and then observed with a light microscope. The fungi identified were confirmed by comparing their morphology and cultural characteristics with descriptions given by Talbot (1971), Deacon (1980), Domoschet et al (1980) and Bryce (1992).

Confirmatory Test for Hydrocarbon Utilization Potentials of The Isolated Fungi

The enrichment procedure as described by Nwachukwu (2000) was used in the estimation of hydrocarbon utilizers. A minimal salt broth 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, 0.10g of $MgSO_4$, $7H_2O$ and 5.0g of agar – agar dissolved in 1000ml of distilled water was prepared. The solution was sterilized by autoclaving. Twenty-eight test tubes were sterilized and placed in test tube racks, where there were four test tube racks containing seven test tubes each. 10ml of the minimal salt broth (MSB) was measured into each of the test tube. 2ml of either crude-oil or diesel or engine oil or spent engine oil or the seed oil extract was measured and added to the 10ml of minimal salt broth in the first 6 test – tubes in each rack respectively with the exception of the last test – tubes, that is, the seventh test tube in each rack, making 12ml in twenty – four test tubes and 10ml of only minimal salt broth in four test tubes which served as controls.

Three fungi which were isolated from *Detarium senegalense* were added to the test tubes in three racks with the test tubes in the last rack serving as control without fungi. Each of the test tubes was 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 incubated at room temperature (28 ^{o}C - 31 ^{o}C) for 40 days. The test tubes were shaken constantly throughout the duration of the experiment to facilitate oil (cell phase contract. The ability to degrade the petroleum products (based on the growth rate of the organisms in the MSB medium) was measured every 5das using the visual method which is based on the turbidity of the MSB. The turbidity was measured using the photoelectric colorimeter. The experiment was carried out twice. The statistical analysis was done using the T – test and anova (F – Test) formula. (Parker, 1979).

Results

Five fungi were isolated from diseased *Detarium senegalense* seeds and they are as follows: *Aspergillus niger*, *Aspergillus flavus*, *Mucor sp*., *Rhizopus sp*., *Talaromyces sp.*

The growth pattern of fungi in minimal salt solution is represented on Figure 1. It shows that the growth rate of each fungus had different maximum growth peaks. There was fluctuation in the growth of each fungus. *Aspergillus niger* had the highest growth rates on the 10th and 30th days with optical densities 4.0 and 3.75 respectively. There was a marked decrease in the growth rates of *Aspergillus niger* and *Rhizopus* on the 15th, 25th and 35th days respectively i.e. every 10 days. *Talaromyces*, earlier observed to be a slow grower when cultured on agar plates had its maximum

growth peak on the 30th day at an optical density of 0.5. On the 40th day, *Aspergillus niger* had the highest growth peak at 1.9, followed by *Rhizopus* at an optical density of 0.275 which was closely followed by *Talaromyces* at an optical density of 0.245.

The growth pattern of fungi in diesel and minimal salt broth is represented on Figure 2. It shows that the growth rate of each fungus had different maximum growth peaks. *Rhizopus* had a maximum growth peak on the 5th day at an optical density of 7.0 while *Talaromyces* had the least growth peak on the 5th day at an optical density of 0.35. On the 35th day, *Rhizopus* attained a growth peak of 6.0 while *Talaromyces* fell to a growth peak of 0.8. On the 40th day, *Rhizopus* had the highest optical density at 5.4, followed by *Aspergillus niger* at 2.15 and *Talaromyces* at 1.1. *Rhizopus* had the highest ability to degrade diesel while *Talaromyces* had the least ability.

The growth pattern of fungi in unspent engine oil and minimal salt broth is shown in Figure 3. *Rhizopus* and *Aspergillus niger* had maximum growth peaks on the 30th day at an optical density of 7.0 with *Rhizopus* earlier having this maximum growth peak on the 5th day and *Aspergillus niger* maintaining this peak on the 35th day. On the 40th day, *Aspergillus niger* had the highest optical density at 6.95 followed by *Rhizopus* at 6.0 and *Talaromyces* at 1.15. *Aspergillus niger* had the highest ability to degrade unspent engine oil while *Talaromyces* had the least ability.

The growth pattern of fungi in spent engine oil and minimal salt broth is represented in Figure 4. *Rhizopus* had a maximum growth peak at optical density 8.0 on the 5th and 10th days respectively. *Aspergillus niger* also had this growth peak at 0.71 on the 5th day. *Talaromyces* had the lowest growth peak at optical density 0.71 on the 15th day. On the 40th day of incubation, *Rhizopus* had the highest growth peak at 6.75 followed by *Aspergillus niger* at 5.25 and *Talaromyces* at 5.0. *Talaromyces* had the best ability to degrade spent engine oil while *Rhizopus* had the least ability.

The growth pattern of fungi in kerosene and minimal salt broth is represented in Figure 5. Rhizopus had the highest growth peak on the 35th day at an optical density of 6.0 while Talaromyces had the lowest growth peak at an optical density of 0.195 on the 10th day. On the 40th day of incubation, *Rhizopus* had the highest optical density at 2.0 followed by *Aspergillus niger* at 1.0 and *Talaromyces* at 0.5. *Rhizopus* had the best ability to degrade kerosene while *Talaromyces* had the least ability.

The growth pattern of fungi in *Detarium senegalense* oil extract and minimal salt broth is represented on Figure 6. *Rhizopus* had a maximum growth peak at optical density 8.0 on the 5th day while *Aspergillus niger* had the lowest optical density at 0.45 on the 35th day. On the 40th day of incubation, *Aspergillus niger* had the highest optical density at 5.5 followed by *Rhizopus* at 3.5 and *Talaromyces* at 2.5. *Aspergillus niger* had the highest ability to degrade the oil extract while *Talaromyces* had the least ability.

The growth pattern of fungi in crude oil and minimal salt broth is represented on Figure 7. *Rhizopus* had the least growth peak at 0.8 on the 5th day. On the 40th day of incubation, *Aspergillus niger* had the highest optical density at 6.5 followed by *Talaromyces* at 6.25 and *Rhizopus* at 5.9.

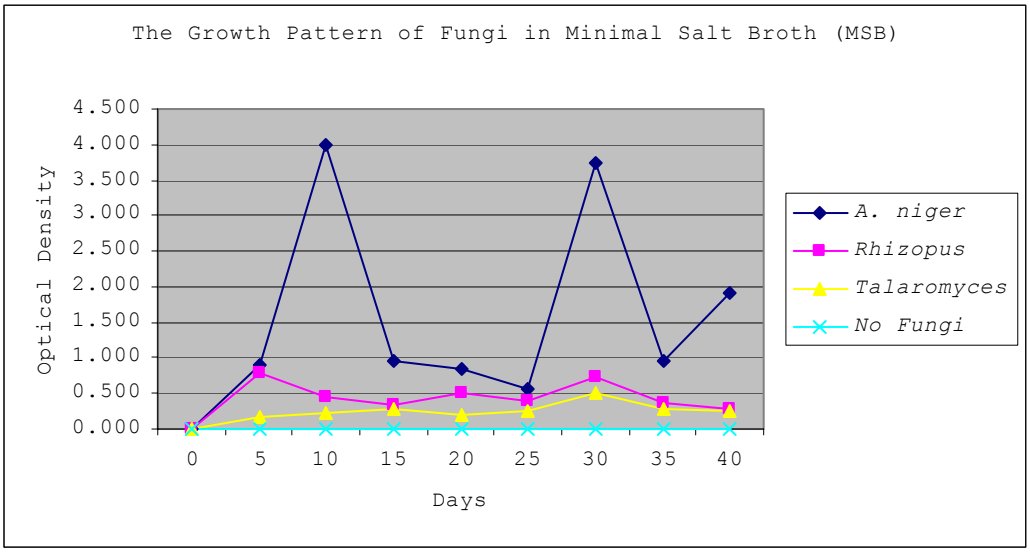

Figure 1: The growth pattern of fungi in minimal salt broth (MSB).

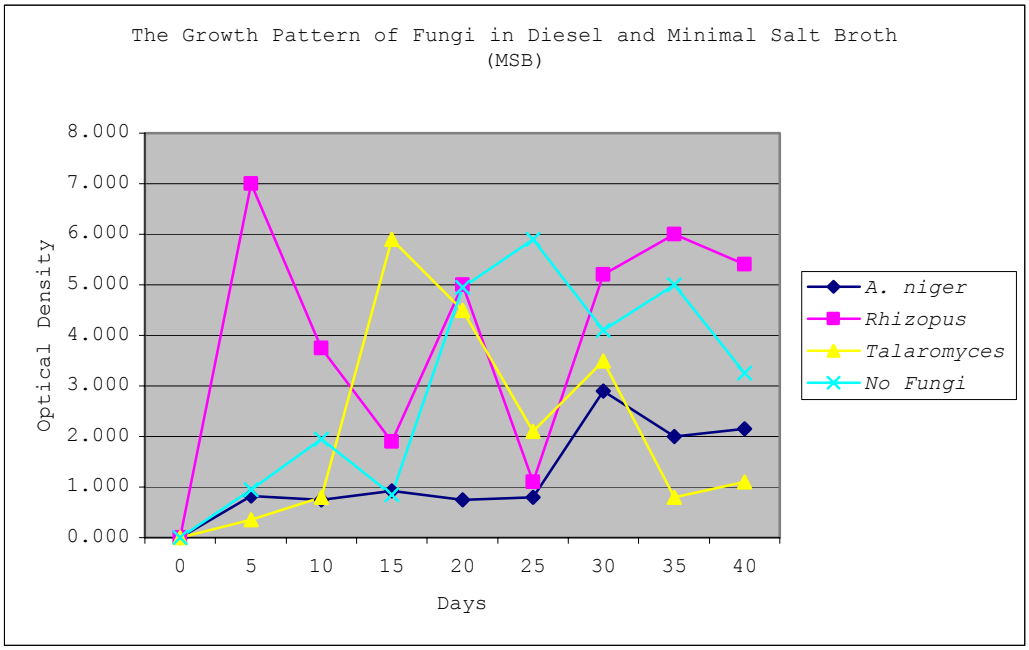

Figure 2: The growth pattern of fungi in diesel and minimal salt broth

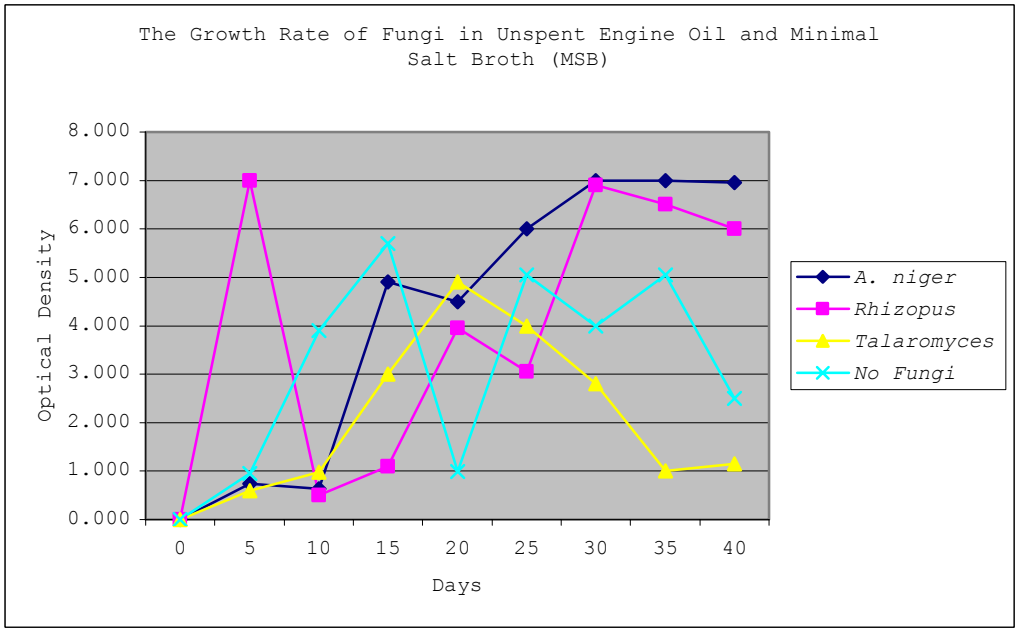

Figure 3: The growth pattern of fungi in unspent engine oil and minimal salt broth.

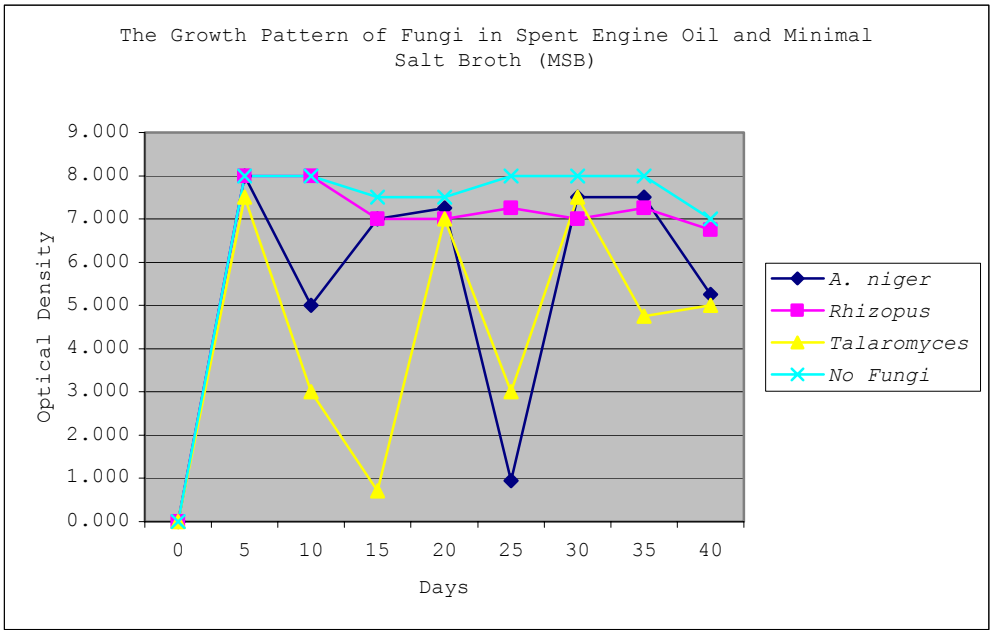

Figure 4: The growth pattern of fungi in spent engine oil and minimal salt broth.

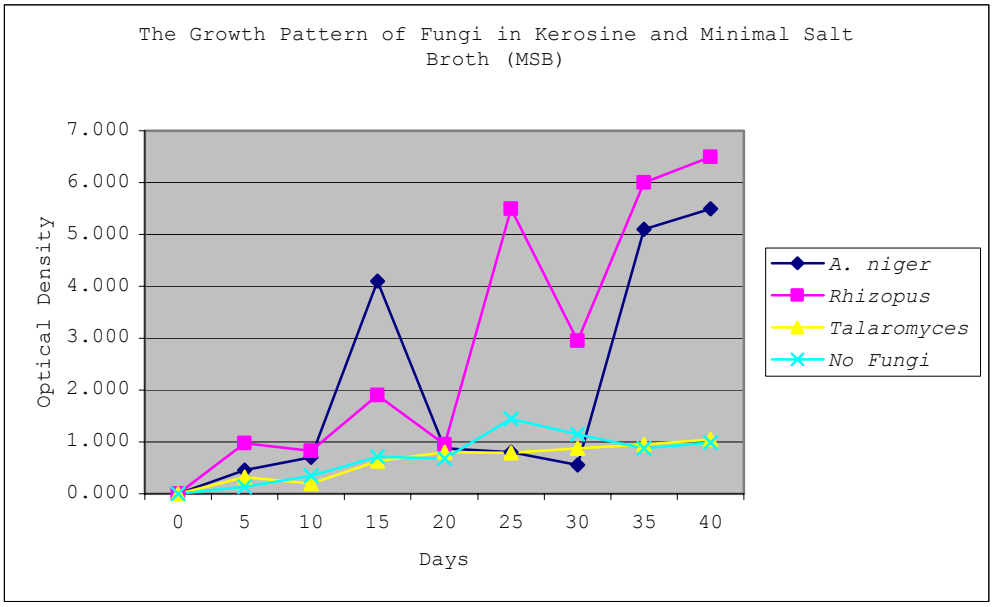

Figure 5: The growth pattern of fungi in kerosene and minimal salt broth

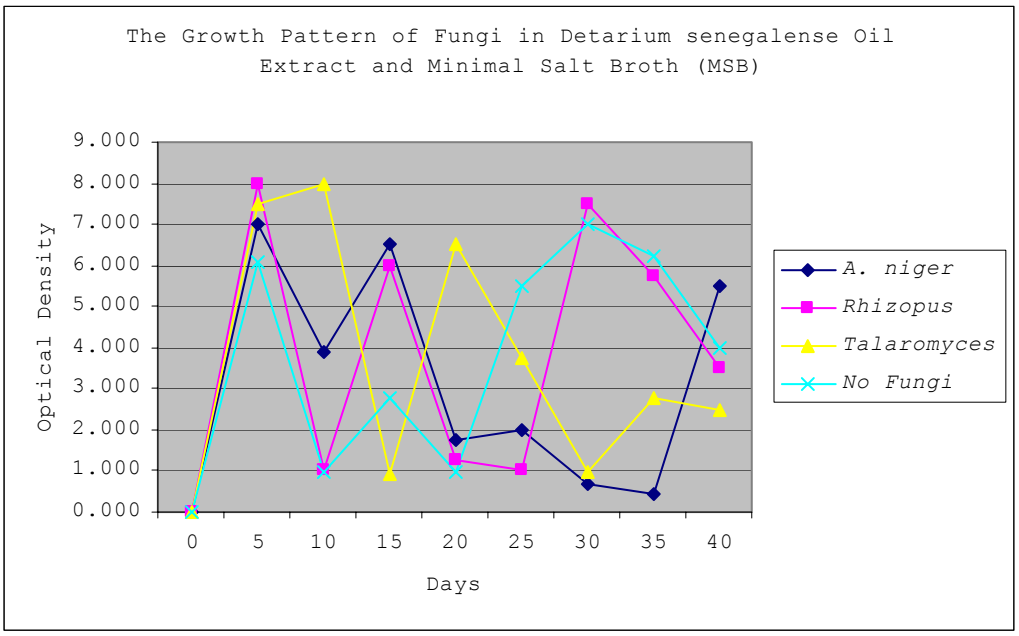

Figure 6: The growth pattern of fungi in *Detarium senegalense* oil extract and minimal salt broth

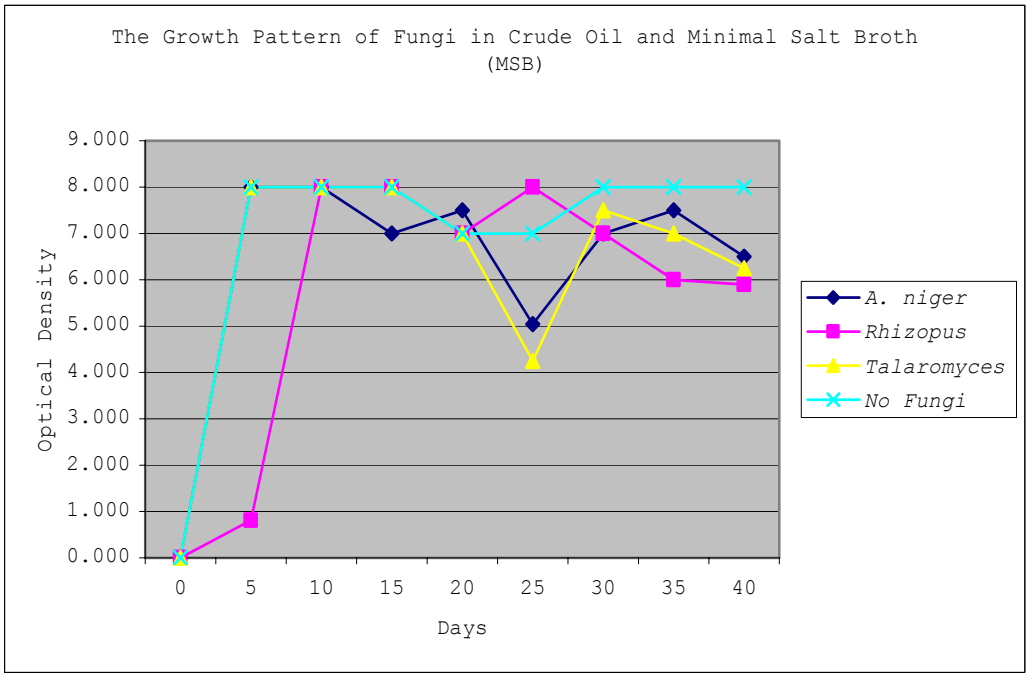

Figure 7: The growth pattern of fungi in crude oil and minimal salt broth.

Discussion

The results of this work indicate that many of the fungal species isolated from the oil seeds were capable of degrading petroleum hydrocarbons. Bartha and Atlas (1973) listed 22 genera of bacteria, 1 algal genus and 14 genera of fungi which had been demonstrated to contain members which utilize petroleum hydrocarbons; all of these micro organisms had been isolated from an aquatic environment. Also, Okerentugba and Ezeronye (2003) demonstrated that *Penicillium spp*., *Aspergillus spp.* and *Rhizopus spp.* were capable of degrading hydrocarbons especially when single cultures were used. These fungi had been isolated also from aquatic environments in the Niger Delta area of Nigeria. Batelle (2000) showed that fungi were better degraders than traditional bioremediation techniques including bacteria. The fungi used were wood-degrading fungi. Also, the ability of the white-rot fungus – *Pleurotus tuberregium* to ameliorate crude oil polluted soil has been reported by Isikhuemhen et al (2003). The isolation of fungal petroleum hydrocarbon utilizers from oil seeds was first documented by Adekunle and Oluyode (2002). Results presented in this study show that the utilization of the different hydrocarbons used, namely, diesel, unspent and spent engine oils, kerosene, crude oil and *Detarium senegalense* seed oil extract vary widely among the fungi isolated. This was probably due to the difference in growth rates of each fungus with each fungi attaining a maximum growth peak and declining after some days, probably as a result of exhaustion of nutrients and release of toxic materials into the medium.

An interesting observation generated in this study was that the fungi isolated had increased growth rates in the media containing petroleum and petroleum products compared to only when minimal salt broth was used. This might be due to the fact that the fungi isolated were able to use the hydrocarbons as substrates for growth by probably releasing extra cellular enzymes and acids which are capable of breaking down the recalcitrant hydrocarbon molecules, by dismantling the long chains of hydrogen and carbon, thereby, converting petroleum into simpler forms or products that can be absorbed for the growth and nutrition of the fungi. Shaw (1995) found that organisms break down hydrocarbons and use the energy to synthesize cellular components. After being completely broken down, the reaction releases Carbon (IV) oxide, water and energy used to create cellular biomass (Keeler, 1996). However, it must be noted that there were also nutrients present in the minimal salt broth though more of it could have been present in the oil which stimulated the growth of each fungus. In view of this then, the additional nutrients present in the minimal salt broth helped in overcoming nutrient limitation to microbial growth to a certain extent and also helped in creating a favourable environment for the rapid development of the fungi especially at the times when the fungi had not started breaking down the hydrocarbons into simpler molecules.

Microbial degradation of oil has been shown to occur by attack on the aliphatic or light aromatic fractions of the oil. Although some studies have reported their removal at high rates under optimal conditions (Rotani et al, 1985; Shiaros, 1989), high molecular weight aromatics, resins and asphaltenes are generally considered to be recalcitrant or exhibit only low rates of biodegradation. Amund and Akangbou (1993) showed that crude oil fractions with lower amount of saturated hydrocarbons were more resistant to microbial degradation than the fraction(s) containing higher amount(s) of saturated hydrocarbons. The Escravos crude oil blend used in this experiment has been shown to contain 69.74% saturated hydrocarbons, 22.05% aromatics, 2.56% asphaltenes and 5.65% residue (Amund and Akangbou, 1993). This could possibly have accounted for the slow rate of degradation of the oil.

In addition, the black colour of the crude oil and spent engine oil samples resulted in the maximum optical density of 8.0 possessed by both samples at the beginning of the experiment. Therefore, the measure of degradation was indicated by a lightness in colour (as the hydrocarbon molecules were broken down) which was equivalent to a reduction in optical density. Therefore, the organism that gave the lowest optical density was taken as the best degrader of both crude oil and spent engine oil in minimal salt broth respectively. 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.

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