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Studies on biodegradation of crude oil by *Aspergillus niger*

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Abstract

Hydrocarbon degrading microorganisms play a major role in the environment. In the present study crude oil degrading fungal strain Aspergillus niger was isolated from oil contaminated soil near crude oil production well (Lingala) Andhra Pradesh. The rate of reduction in some petroleum hydrocarbon fractions, such as n-alkanes, aromatics, nitrogen, sulfur and oxygen (NSO)-containing compounds and polycyclic aromatic hydrocarbons (PAHs), were monitored by means of gas chromatography. The nC_{17}/Pristine and n C_{18}/Phytane ratios, extrapolated from the GC profiles decreased from the initial value of 2.510 and 7.289 to 0.132 and 0.474 respectively at day 60.

Keywords: *Crude oil, Aspergillus niger, Biodegradation, nC_{17}/Pristane, n C_{18}/Phytane.*

1. Introduction

As the oil dependency is increasing the allied problems are becoming more and more cumbersome. Since 1920's, oil and gas exploration and production have increased to enormous proportions in developed and developing countries (United States, Europe, Middle East, Far East, Africa and South America). World crude oil production is about 60 million barrels/day (b/d). In 1990's it was estimated that 16.4 million barrels or 0.07 % of the total world oil production (21.9 billion barrels/year) might have spilled from all sources, including natural seepage, production, storage, transportation, and manufacturing of oil and oil products (Salanitro, 1992).

The use of fungi as a method of bioremediation provides an option to clean up environmental pollutants. Bioremediation using fungi have drawn little attention in the past two decades since most of the bioremediation researches focused mainly on the use of bacteria. Nevertheless, recently fungi have received considerable attention for their bioremediation potential that is attributed to the enzymes they produced that are involved in lignin breakdown which degrade a wide range of recalcitrant pollutants such as polyaromatic hydrocarbons, chlorophenols, and pesticides (Bumpus *et al.*, 1985). Bartha and Atlas (1977) listed 22 genera of bacteria, 1 algal genus and 14 genera of fungi which had been demonstrated to contain members which utilize petroleum hydrocarbons. Batelle (2000) showed that fungi were better degraders than traditional bioremediation techniques including bacteria.

Elshafiea *et al*. (2007) showed that *Aspergillus* and *Penicillium* species were more active than the others Molla *et al*. (2002) reported that the strains/isolates *Aspergillus niger* , SS-T2008, WW-P1003 and RW-Pl 512 produced the highest dry biomass at higher sludge supplemented culture media from their respective group (*Aspergillus*, *Trichoderma*, *Penicillium* and *Basidiomycetes*, respectively).

2. Materials and Method

2.1 Sample Collection and Isolation

The organism used in this study was isolated from soil contaminated with crude oil at crude oil production site of O.N.G.C. from oil well of Lingala oil field project, Andhra Pradesh. A fungal isolate designated as *Aspergillus niger*, was isolated from oil contaminated soil by plating out low dilutions (10^{-1} to 10^{-2}) of sample on to mineral salt medium (Mills *et al.*, 1978). The medium has the following composition [in (g/l)]: NaCl (10), $MgSO_4.7H_2O$ (0.42), KCl (0.29), KH_2PO_4 (0.83), Na_2HPO_4 (1.25), $NaNO_3$ (0.42), Agar (15), distilled water (1000 ml), and pH (7.2). The medium was autoclaved (subject to pressure sterilize at 121^{o}C) at 1.1 kg/cm2 for 15 min. The inoculated mineral agar plates were then inverted over sterile membrane filters, moistened with crude oil (Lingala well) and held in the lid of the petri dishes. The petri dishes were wrapped with masking tape so as to increase the vapor pressure within the dishes during incubation of the petri dishes at 29º C for 7 days. After incubation, the fungal cultures were stained with methylene blue and observed under a high-power resolution microscope (x 40). Fungal culture was identified based on its morphological characteristics. It was maintained on Czapek dox agar medium at 2 °C with sub-culturing every two months.

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 the maximum diameter (9 cm) of the petri dish and the texture of fungal growth were observed. After 2-4 days of the 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).

2.1 Biodegradation Studies and TPH (Total Petroleum Hydrocarbons) Extraction

Growth and degradation studies over a time course were carried out using crude oil of the Lingala well as the sole carbon and energy source. The crude oil used for the study had an initial composition of Sat % 67.3; Arom % 21.2 %; NSO % 11.2; Asp % 0.3. Mineral salt medium (200 mL) was prepared in 4 Erlenmayer flasks (1000 mL capacity). Starter culture (CDB-grown mycelium, 96h, 30ºC, 3.4g dry weight) was harvested by centrifugation at 6000xg for 20 min, suspended in mineral salt medium and crude oil (2.4 g) as the sole carbon and energy source was added in each mineral salt medium (200 mL) flask. The flasks were covered with non-absorbent cotton wool and

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placed in a slanted position to allow air passage through the pores of cotton wool. The flasks were shaken manually at regular intervals to allow adequate mixing and homogeneity of the contents. The experimental setup was monitored for a period of 60 days. After 15 days of time interval, the flask was taken out and microbial activities were stopped by adding 1% 1N HCl. For extraction of crude oil 50 mL culture broth was mixed with 50 mL petroleum ether : acetone (1:1) in a separating funnel and was shaken vigorously to get a single emulsified layer. Acetone was then added and shaken gently to break the emulsification, which resulted in three layers. Top layer was a mixture of petroleum ether, crude oil and acetone; clumping cells make the middle layer and the bottom aqueous layer contains acetone, water in soluble form. The lower two layers were separated out while top layer containing petroleum ether mixed with crude oil and acetone was taken out in a clean beaker. The extracted oil was passed through anhydrous sodium sulphate to remove moisture. The petroleum ether and acetone was evaporated on a water bath. The gravimetric estimation of residual oil left after biodegradation was made by weighing the quantity of oil in a tared vial. The biodegraded crude oils were further fractioned for their gross and molecular composition.

2.1 Fractionation of TPH and Analysis

TPH was fractionated into alkanes, aromatic, asphaltene and NSO fractions on silica gel column (60-120 mesh). The column was washed with petroleum ether. The oil sample (approx. 100 mg/known weight) was dissolved in chloroform, adsorbed on silica gel and evaporated the excess chloroform at 80 °C. The adsorbed sample was charged at the top and eluted saturates with 60 mL petroleum ether (40-60 °C), aromatics with 90 mL benzene and NSO with 60 mL methanol respectively. The solvents were evaporated in rotary vacuum evaporator at 60 °C. The alkane and aromatic fractions of fresh and degraded oil were analyzed by gas chromatography using chemito-1000 gas chromatogram, equipped with a single flame ionization detector (FID). For alkane fraction BP-5 capillary column of 30 m length and an internal diameter of 0.25 mm wide bore of 1 µ film thickness were used. A temperature program of 80-300 ºC, increasing at 4 ºC per minute for 30 min was employed. Nitrogen with a flow rate of 40ml/min was used as a carrier gas. The Injection port temperature was 310 °C. The oil extracts of culture supernatants were dissolved in methylene chloride, while a sample volume of 0.2 µl was injected. The nC_{17}/Pristane and nC_{18}/Phytane ratios were subsequently calculated from the height of various chromatograms. Conditions for aromatic analysis, Column, BP-5 (60 m X 0.25 mm); Initial temperature, 80°C; Final temperature, 300 °C; Ramp rate, 3 °C per min; Carrier gas, Nitrogen (30 mL/min); Injection temperature, 310 °C.

3. Results and Discussion

The fungal isolate used in this study *A niger* was the predominant fungal isolate found in soil contaminated with crude oil at crude oil production site of O.N.G.C. from oil well of Lingala oil field project, Andhra Pradesh. It has been reported in the literature that a significant number of soil fungi utilize petroleum hydrocarbons very efficiently, though slowly (Cerniglia *et al.,* 1980). The filamentous fungi can grow on hydrocarbons, with *Aspergillus* and *Penicillium* species being the most frequently reported (Colombo *et al.*, 1996; April *et al.*, 2000; Chaillan *et al.*, 2004).

Aspergillus niger when grown on Czapek dox agar medium shows dark brown velvety growth with black conidial heads. On reverse side colony is pale yellowish. When observed under light microscope, the conidia appeared to be well arranged and spore chains were formed after incubated for a period of 1 week.

The ability of the fungal isolates in degrading crude oil was determined by the morphological changes on the fungal isolates, the enzymatic activities of ligninolytic enzymes secreted by the fungal isolates (data not shown) as well as the gas chromatography with flame ionization detector (GC-FID) for their ability to degrade hydrocarbon in minimal salt medium amended with 1% (v/v) crude oil. The untreated crude oil of Lingala was used as a reference, had an initial nC_{17}/Pristane and nC_{18}/Phytane ratios of 2.510 and 7.289, respectively. Figure 1 (a) shows the results of GC-FID of control using untreated crude oil and Figure 1 (b) shows the GC profile of residual crude oil for *Apergillus niger* isolate conducted after incubation period of 2 months. The saturate profiles of the fractionated residual oil showed that n-alkanes have been removed from crude oil incubated with isolate. They show predominance of odd and even normal alkanes between C_6 and C_{37}. By comparing the GC profiles for the different days, it was observed that there was a significant decrease even at 10% probability level in the peaks corresponding to aliphatic hydrocarbon fractions of the oil. According to April *et al.* (2000), the reduction in peak heights of the pristane and pyrene indicates the degradation of the branched alkanes isoprenoids components. This is well observed in all of the saturate profiles showing that the fungal isolates are capable of degrading the branched alkanes isoprenoids. This study supports the findings by April *et al.* (2000) that reported 22 species of *Penicillium* and 5 species of *Aspergillus* isolated from the flare pit soils in Northern and Southern Canada showed the ability to degrade hydrocarbons on solid medium amended with crude oil.

The biomarker parameter, nC_{17}/Pristane decreased from 2.510 to 0.132 after 60days of treatment. Similarly, nC_{18}/Phytane ratio is also decreased from 7.289 to 0.474 in 60d. The ratio of Pr/Ph has not changed drastically and shows a meager degradation of pristane. The GC profiles and the biomarkers such as the nC_{17}/Pristane and nC_{18}/Phytane ratios which showed significant reductions over the period are evidence indicating gradual decontamination hence recovery of the site (Teal *et al., 1992*; Wang *et al.*, 1994; Yveline *et al.*, 1997; Nwachukwu *et al.*, 1998).

Gas chromatogram of alkane fraction of crude oil biodegraded by *Aspergillus niger* indicate extensive biodegradation in 60 d period. The ratios of the isoprenoids, pristane and phytane increase to the North West suggesting depletion of normal paraffins (12). Gas chromatograms of aromatic fraction of *Aspergillus niger*.

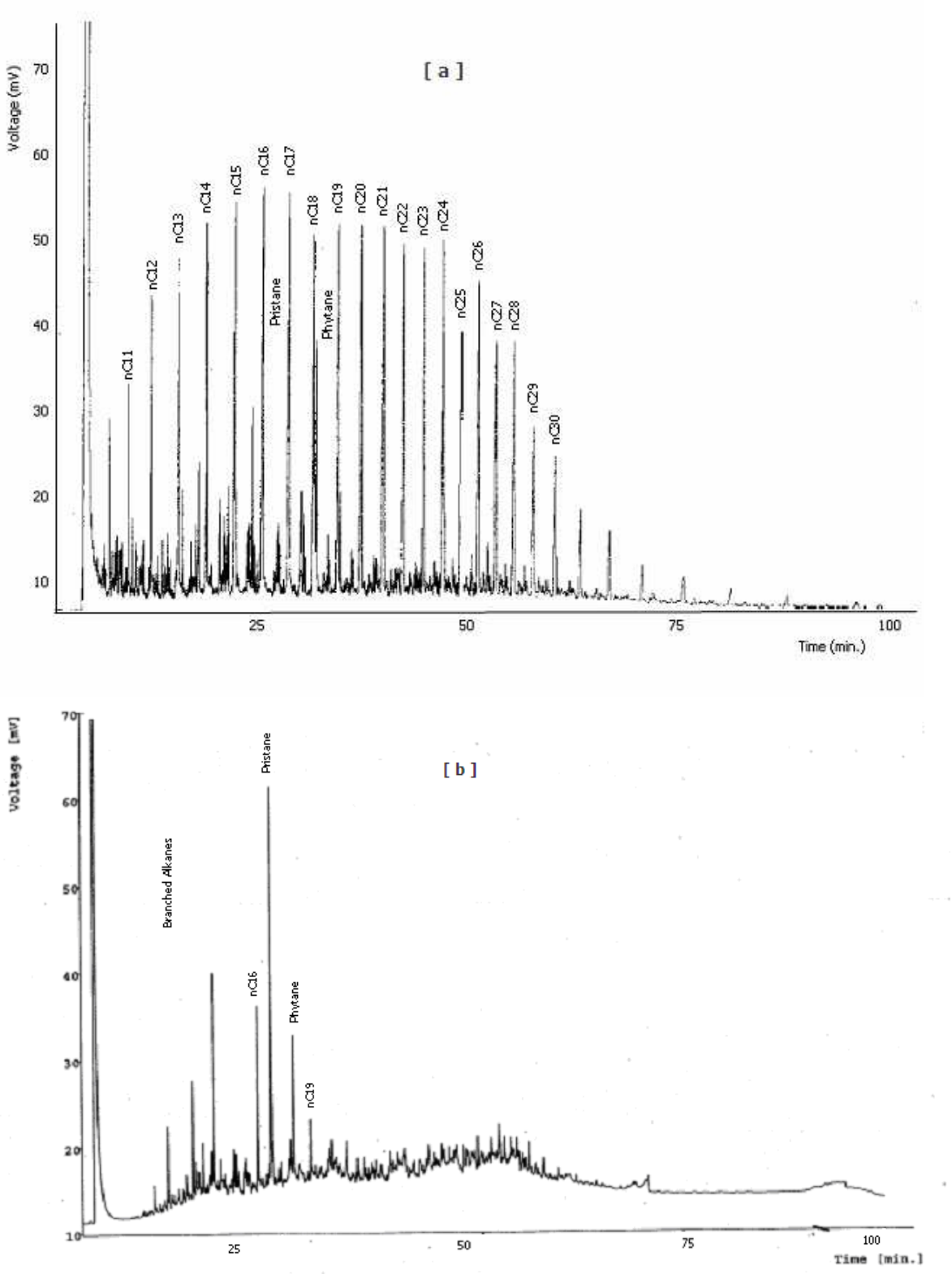

Figure 1. Gas chromatographic fingerprinting of (a) initial (zero day) saturate fraction of crude oil and (b) after 60days of bioremediation of saturate fraction of crude oil by Aspergillus niger.

revealed that the lower aromatic fractions have been removed over a period of time and higher aromatic hydrocarbon abundance is seen. It is evident that fungi have a greater capacity and enzymatic capability to degrade the recalcitrant PAH than bacteria. Other researchers, (e.g. Andrea *et al.*, 2001; Cerniglia, 1992;

Gadd, 2001 and Sutherland, 2004) have reported that fungi are good PAH degraders.

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

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