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ISOLATION OF OIL EATING FUNGI FROM PETROLEUM CONTAMINATED AREAS OF THE THAR DESERT

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ABSTRACT

In present study, soil samples were collected from petroleum contaminated areas of the Thar Desert for the isolation of oil eating fungi. Initial assessment of the oil degrading fungi was done using mineral salt medium (MSM) supplemented with crude oil as the sole carbon source. Fungal counts were obtained from each of the samples and a total of seven fungal isolates were chosen. Their ability of utilizing crude oil was detected using vapor-phase transfer assay. Isolate F2 showed the maximum growth whereas isolate F7 showed the minimum growth. After morphologically identifying the fungal strains their viability assay was done using Fluorescence diacetate FDA hydrolysis Assay. Maximum viability of the mycelia was observed for strain F2, followed by F4 and F5 respectively, in the form of fluorescence under UV radiations.

KEYWORDS: ***Crude oil, FDA assay, Mineral Salt medium (MSM), Viability***

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INTRODUCTION

India is a developing country and in the past few decades it has emerged out as a hub of development and industrialization on the map of the world. To meet the demands of the people rapid expansion of industries, food, healthcare and vehicles are inevitable. But it is very difficult to maintain the quality of life with all these new developments which are unfavorable to the environment. Oil spills occur due to leakage from points of the petroleum oil pipelines, accidents of oil tankers during transportation of crude oil, anthropogenic and pilferage activities [2] The Thar Desert is a large, arid region lying in the northwest of the Indian subcontinent. It owes multiple oil drilling sites and petroleum plants [3]Petroleum crude oil is a complex mixture of hydrocarbons ranging from simple straight and chained alkanes to complex polycyclic aromatic hydrocarbons (PAHs). In view of this chemical complexity, the refinery of crude oil yields a diverse range of useful products such as liquefied petroleum gas, diesel, lubricating oils, bitumen and chemical solvents [4]Pollution results in the deterioration of both biotic and abiotic components of the ecosystem as some hydrocarbon components have been considered belonging to families of carcinogenic and neurotoxic organ pollutants [5.] Excavation, secured landfill disposal, thermal desorption, incineration, stabilization and solidification are some of the techniques that can be used to treat oily sludge contaminated soil [6]but after treatment contaminants produce some by-products that have negative impacts on the environment as well as public health [7] Biological agents like microorganisms or plants transform the complex organic contaminants into other simpler organic compounds owing to their diverse metabolic capabilities for the removal and degradation of environmental pollutants[8]. The US EPA has defined bioremediation agent as “Microbiological cultures, enzyme additives, or nutrient additives” that significantly increase the rate of biodegradation to mitigate the effects of the discharge[9] The remediation of polluted soils in desert region requires the study of the microorganisms’ diversity in the environment and the determination of the ability of different microbes and their consortia to degrade pollutants in the presence of high salt concentration [10]Bioremediation promotes the microbial metabolism of contaminants by adjusting the water, air and nutrient supply in the soil [11]The reported efficiency of biodegradation ranged from 6% [12]to 82% [13]for soil fungi, 0.13% [12]to 50% [13]for soil bacteria and 0.003% [14]to 100% [15]for marine bacteria.

Mycoremediation

it is fungi that can especially handle breaking down some of the largest molecules present in nature [16]The main function of fungi in the ecosystem is decomposition, which is performed by the fungal mycelium. The fungus secretes a number of extra cellular enzymes and acids that break down lignin and cellulose, which are organic compounds composed of long chain of carbon and hydrogen present in the plant fibre which is similar in structure to many organic pollutants thus fungi can be used to degrade these pollutants. Filamentous fungi are more advantageous where translocation of essential factors (nutrients, water, the pollutant itself) is required for the degradation of environmental chemicals by translocating resources between different parts of their mycelium. Extracellular enzymes secreted by fungi cause digestion of energy sources in their surroundings and further diffusion of these molecules through the substrate towards the fungus [18]Fungi are also known to produce large quantities of exudates that serve as auxiliary carbon sources for pollutant-degrading bacteria. Fungi degrade PAH more than bacteria. Fungi can degrade high molecular weight PAHs, whereas bacteria are best at degrading smaller molecules. Keeping in view the comparatively higher biodegradation potential of fungi than bacteria towards hydrocarbons; our current study aimed at isolation of hydrocarbon utilizing fungi from the oil contaminated areas of the Great Indian Thar Desert.

MATERIALS AND METHODS

Collection of the Soil Samples

Sampling sites were investigated in the arid region of the Thar and a total of six soil samples were collected. Surface was dug upto 1cm depth and soils were kept in pre sterilized bags, kept at 4ºC prior to experimentation. The samples were labeled accordingly (SP1-SP6). Standard microbiological procedures were employed in the collection and handling of the soil samples and analysis was done within 24 hours of collection.

Medium for Petroleum Utilizing Fungi

For the isolation of oil utilizing fungi oil agar plates were prepared using Mineral Salt Medium (MSM)[20]; modified by [21]The composition of the medium was NaCl, 10.0 g; $MgSO_4.7H_2O$, 0.42 g; KCl, 0.29 g; KH_2PO_4, 0.83 g;Na_2HPO_4, 1.25 g; $NaNO_3$, 0.42 g; agar, 20 g; distilled water, 1 L; pH -7.2. The medium was used for isolation, enumeration and preliminary identification of petroleum-utilizing fungi (oil-degraders).1 % (v/v) crude oil was sterilized with 0.22 µm pore size Millipore filter paper Moslein France [22]and was added into sterile MSM, which after cooling it to 45 °C under aseptic condition. Tetracycline was added to prevent bacterial growth. The MSM and crude oil were then mixed thoroughly and dispensed into petri dishes.

Isolation and Enumeration of Petroleum Utilizing Fungi

The soil samples were subjected to serial dilution technique and inoculated on these oil agar plates. Sterile physiological saline, i.e. 0.85 % (w/v) sodium chloride was used as diluent for inoculum preparation. 1.0 g of homogenized, 2 mm sieved soil sample was aseptically transferred into a sterile test tube containing 9.0 ml of the diluent giving 10^{-1} dilution. Subsequently, three-fold (10^{-3}) serial solutions were prepared from the 10^{-1} dilution. 0.1 mL aliquot of 10-3 dilution of each soil sample was aseptically inoculated to oil agar plates via pour plating method. Agar plates were incubated for 5-7 days at room temperature (28 - 30ºC). The fungal population in each sample was expressed as colony forming units (CFU) that determined an estimate of viable mycelia. Plate counting

aimed at confirmation that every colony is separate and formed by single viable cell.

Characterization of Fungi

On the basis of morphological characters such as size, shape, color of spores, formation and texture, the fungal strains were characterized by mounting and examining under microscope.

Confirmatory identification of true petroleum utilizing fungi by Vapor-phase technique

Crude oil utilization test was carried out for the confirmatory identification of actual petroleum-utilizing fungi choosing seven isolates obtained from the oil agar preliminary isolation medium The composition and preparation of the crude oil utilization test medium was the same as that of oil agar medium except that oil was made available via vapour phase transfer [23]Putative petroleum-utilizing fungal isolates were individually streaked on plates of agar medium. On the inner side of the petridish lid; a sterile Whatman, No. 1 filter paper saturated with filter-sterilized crude oil was placed. This aimed at supplying hydrocarbons as sole sources of carbon and energy for the growth of the microorganisms on the mineral salts agar medium surface through vapour- phase transfer. All the plates were inverted and incubated at 28 ± 2 °C for 7-14 days [24]Uninoculated plates served as control.

Fluorescein diacetate (FDA) hydrolysis assay for Viability of Oil Eating Fungi

Fluorescein diacetate *(FDA)* hydrolysis assay was used to measure enzyme activity produced by microbes in a sample. A bright yellow glow is produced and is strongest when enzymatic activity is greatest. Living cells actively convert the non-fluorescent FDA into the green fluorescent compound "fluorescin" as a sign of viability. In view of determining the ability of isolated fungal strains for utilizing hydrocarbons as a sole energy source FDA assay was used. Bushnell-Haas broth medium was used for the screening test which composed of: $MgSO_4$ (0.2 g/l), $CaCl_2$ (0.02 g/l), KH_2PO_4 (1 g/l), KH_2PO_4 (1 g/l), $FeCl_2$ (0.05 g/l) and NH_4NO_3 (1 g/l) and crude oil (1%) as the sole carbon source (pH-7.0) [25]2.8 ml of BHM was dispensed into sterile petri plates and each was inoculated with 0.2ml MSM culture of fungal strains in replicates. Plates were incubated for 3-5 days at 28.0 ± 1.0°C. FDA stock solution was prepared in acetone as (5mg/1ml Acetone) and was stored in dark at 4 ºC. After the completion of incubation period 2ml of FDA solution was added in each of the incubated plates. The plates were visualized under Ultra violet trans-illuminator for the appearance of fluorescence by viable cells.

RESULTS AND DISCUSSION

Isolation and enumeration of petroleum utilizing fungi

The microbial population in a sample was expressed as colony forming units (CFU) that determined an estimate of viable cells of microorganisms. Plate counting aimed at confirmation that every colony is separate and formed by single viable cell. Plate counts (cfu/g) calculated for each of the six petroleum contaminated soil samples are shown in *fig.1.* The highest plate counts of fungi were obtained from sample S2 as $6X10^6$.

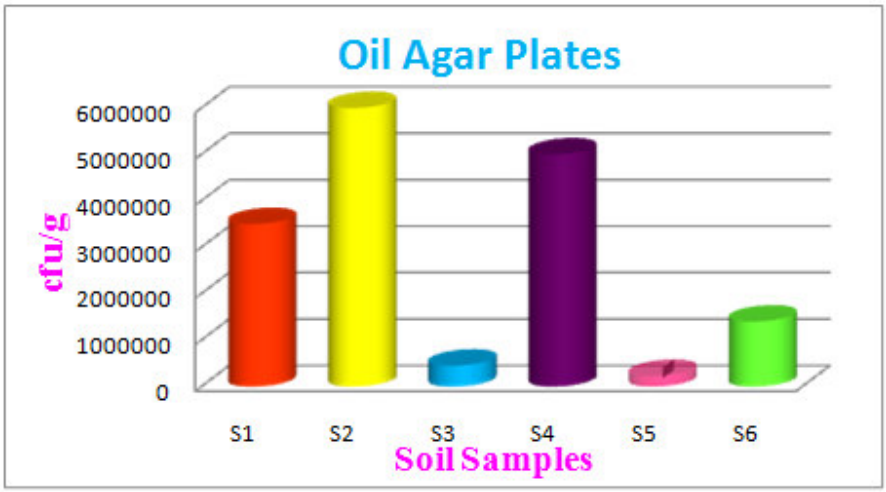

Figure 1
A total of seven fungal isolates were selected on the basis of morphology and color by mounting and examining mycelia under microscope.

Confirmatory identification of true petroleum utilizing fungi by Vapor-phase technique

The ability to utilize oil as a sole source of carbon is shown in Fig. 2 with respect to the fungal counts of each of the seven isolates. Isolate F2 showed maximum growth utilizing the oil via vapor phase transfer followed by Isolate F5. Minimum growth was shown by isolate F7 depicting its poor oil utilizing capability. This assay determined the ability of six fungal isolates as an ideal degrader of petroleum and its products.

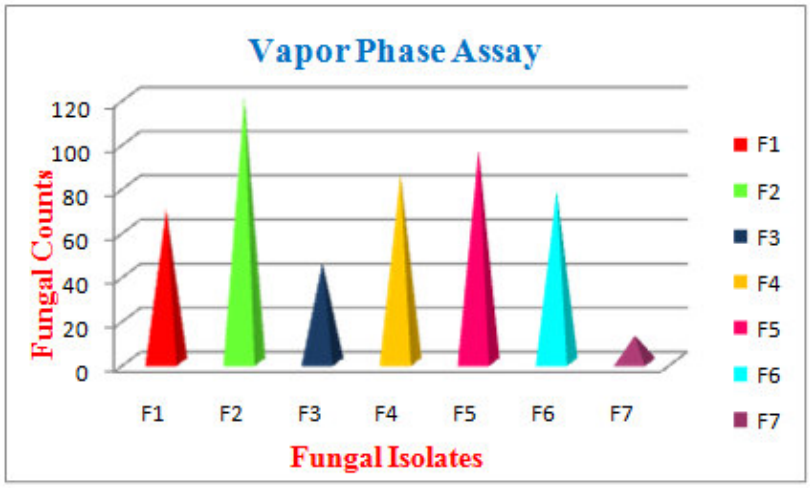

Figure 2

fluorescein diacetate (fda) hydrolysis assay for viability of oil eating fungi

FDA assay confirmed the viability of each of the six fungal isolates by the production of fluorescence under UV rays. Figs. 3-6 show the different intensities of fluorescence produced by fungi depending upon their ability to utilize oil as a sole energy source for their growth.

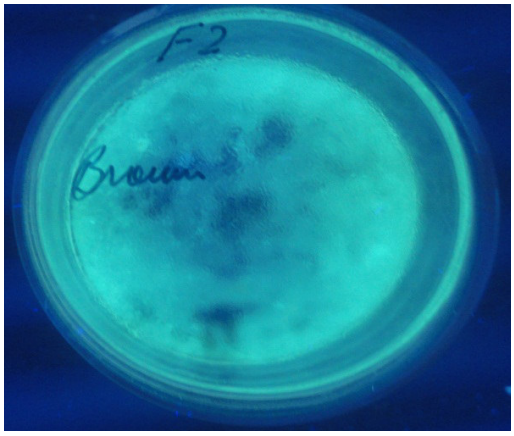

Figure 3

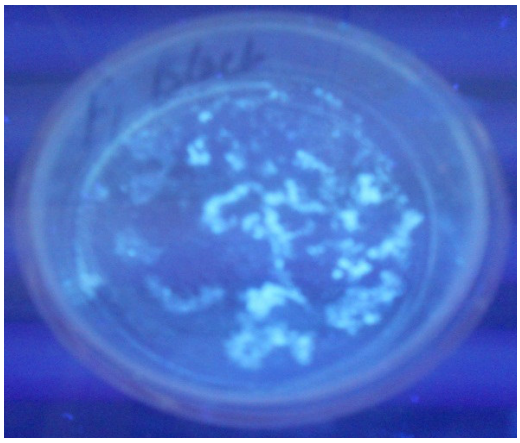

Figure 4

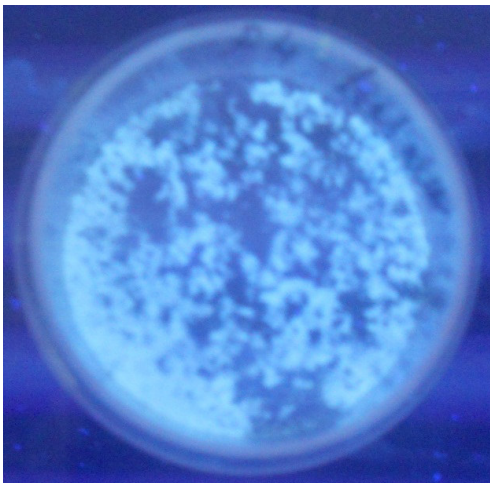

Figure 5

Figure 6

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