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Research article

BIODEGRADATION OF PETROLEUM HYDROCARBONS POLLUTANTS IN SOIL USING MICROBIAL CONSORTIUM

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ABSTRACT: Soil samples were collected from oil contaminated sites at Coimbatore. MTCC cultures, *Micrococcus* sp., *Bacillus* sp., *Pseudomonas* sp, which are able to utilize the oil in soil as carbon source, were added to oil contaminated soil samples. The growth profiles were determined by monitoring the optical density, dry weights and pH of the culture utilizing lubricating oil as sole carbon and energy source. They were incubated at 30˚C for one week. Optimization was done based on the pH, temperature and nutrient contents. The microbial consortium showed optimum growth in day 21. The optical density varied from 0.543- 7.279. The gravimetric analysis showed the microbial consortium was capable of utilizing 80-90% the oil samples, under laboratory conditions at 30˚C and 170rpm with Bushnell-Hass medium. Correlation analysis indicated positive correlation between optical density and pH (correlation coefficient = 0.735). After the extraction, the quantity of hydrocarbon content present in the soil sample was determined using GC- MS. Gas chromatographic analysis showed the degradation for petrol, diesel, and kerosene are 48%, 78% and 57% respectively. The statistical analysis was done with Students T-Test with $p<0.05$.These can be applied to oil contaminated area for cleanup process.

Key words: hydrocarbon, pollution, biodegradation, microbial consortium

INTRODUCTION

Contaminated soils are threat to the human and the ecosystem. Biodegradation of such contaminated soils is required by convention method that is affordable and eco friendly [1, 2]. Many bacteria are capable of utilizing and degrading the oil constituents in a better way [8]. Petroleum hydrocarbons are not easily degradable. Microorganisms have the capacity to degrade majority of hydrocarbon components, the saturated and unsaturated alkanes, monoaromatic and low molecular weight polycyclic aromatic hydrocarbons (PAHs). The organism must be in contact with their substrate to utilize and degrade the hydrocarbon. Individual microorganisms can metabolize only a limited quantity of hydrocarbon substrates. So the mixed cultures of microorganisms are required to increase the rate of petroleum biodegradation [19]. When the organism grows in the contaminated soil, they utilize these constituent hydrocarbons as substrates. Such hydrocarbon utilizing microorganism shows emulsifying activity [3, 4]. MTCC bacterial culture *Pseudomonas* sp, *Bacillus* sp, *Micrococcus* sp, were used as oil degrading organisms. These microbial consortia use the hydrocarbon as substrates and degraded it. These organisms have high capability of degrading the hydrocarbons. The majority of hydrocarbons are found in Diesel, Petrol, and Kerosene. The degradation rate is affected by several physico, chemical and biological parameters such as pH, temperature, nutrient, quantity of hydrocarbon [16]. The hydrocarbon presented in contaminated soil samples were analysed using GC-MS. The biodegradation of the hydrocarbons are challenging as they are complex in structure. The use of detergents as a hydrophobic compound for the hydrocarbon makes it possible for the microorganism to use as a substrate. These make them useful in land farming.

MATERIALS AND METHODS Soil collection and analysis

The contaminated soil sample was dried, homogenized, sieved for the analysis of the hydrocarbon in it. The pH and moisture content of the soil was determined. The soil samples were divided based on the oil present in it, Diesel, Kerosene, and Petrol.

These three samples were added with the required nutrient, carbon, nitrogen, phosphorus. During the study period additional oil (Contaminated soil + Petrol, Diesel, Kerosene) was added to the contaminated soil samples after 7 days, 14 days, 21 days interval.

The samples were evenly spread to enhance the aeration after each nutrient supplementation. The contaminated soil samples were covered with aluminium foil and incubated at 30˚C [15]. The mixture was sampled weekly to determine the amount of hydrocarbon degraded and bacterial population.

Enrichment

Bushnell – Hass medium was used to isolate the hydrocarbon-degrading microorganism from soil sample obtained from contaminated site. 5 gm of soil samples was transferred to Erlenmeyer flask containing 100ml of Bushnell-Hass medium with 250ul of 40% glucose solution, 10%(v/v) of oil as carbon source and 1ml of each microorganism. Bushnell-Hass medium contains 0.4g of KH_2PO_4, 0.4g of $K_2H PO_4$, 0.4g of $(NH_4)_2 SO_4$ or NH_4NO_3, 0.002g of $FeCl_3$,

0.4g glucose. The flasks are incubated at 30˚C on a rotary shaker at 170rpm for 7 days. After 4 days, 1.0ml of the culture was transferred to fresh media containing 1% crude oil and reincubated at 30˚C (Thenmozhi *et al.*, 2011).

Characterization

The incubated media was centrifuged, cell pellet were washed and resuspended in Bushnell – Hass medium. The supernatant was measured in spectrometer with OD 600 until the value was equivalent to 1. These medium was optimized by addition of the nutrients, determining the pH and temperature. Nutrients such as Phosphate, Nitrogen (Ammonium nitrate, Ammonium sulphate, Sodium nitrite, Sodium nitrate and Urea) Carbon sources, Temperature (25, 30, 35, and 40) and pH (5, 6, 7, 8, 9) were used to enhance the degradation rate of the hydrocarbon present in the medium. The growth was observed by measuring the optical density at 600nm and total viable count (cfμ/ml) of the isolates were determined by plating technique on the nutrient agar plates at 30˚C for 24hrs [19].

Dry mass determination

Dry weight mass is the biomass obtained from the substrate. The filtered substrate was dried in oven at temperature 80˚C for 1 hour and cooled in desiccator; repeating until there were no further weight changes. These dry weight mass is determined as the metabolite utilized by the organism.

Extraction

Soxhlet extraction

Soil samples (10g) were extracted with the hydrocarbon present in it. They were air dried for 24hrs in oven at 60˚C, ground, homogenized and sieved. The mixture was placed on Whatman cellulose thimble. Conical flasks were pre-weighed in grams prior to the gravimetric analysis. The soil samples were extracted using 120ml Dichloromethane (DCM) for 24 hrs at a rate 6 cycles per hour in a Soxhlet extraction apparatus [9]. The DCM fraction was collected and evaporated using a rotary evaporator at 40˚C. The percentage of degradation was determined using the amount of remaining Oil with the oil in 10g contaminated soil sample at day 0 as 100% [15].

Biosurfactant extraction

Biosurfactant was extracted from the culture broth by centrifugation of the cell free culture broth at 10000 rpm at 4˚C for 30 mins. The supernatant was alkaline, so it was adjusted to acidic pH 2 by adding 1M Sulphuric acid (H_2SO_4). Biosurfactant extraction was done using equal volume of chloroform-methanol (2:1) mixture. This extracted organic phase was evaporated, dried at 60˚C to get a constant weight [3].

Gas chromatography – mass spectrophotometry

100g of contaminated soil sample were analyzed by GC/MS to determine the quantity and composition of the total hydrocarbon.

Emulsification activity:

The emulsification index (E_{24}) [6] was measured for checking the stability of the extracted biosurfactant. Emulsification activity was measured by adding 2ml of the oil substrate to 2ml of extract and vortexes for 5 mins, measured after 24hrs.

Growth of microbes using hydrocarbon as substrates

Microbes use the hydrocarbon as substrates. Based on the optimum Temperature, nutrients and pH they are able to grow rapidly and degrade it [13].

Detergents

Hydrocarbons are hydrophobic in nature and are strongly absorbed to the soil, especially to the soil organic matter. These make them difficult to degrade. Detergents are used to desorb the hydrocarbon. These make the surface of the bacteria hydrophobic. The hydrocarbon clings to the detergent which makes the bacteria to degrade them easily [8].

Statistical significance

All values were presented as the mean ± the standard deviation. Student t-tests were used to examine the statistical significance (SPSS version 13) between different oil degradation with various parameters. Probability was set at 0.05.

RESULTS AND DISCUSSION

Hydrocarbons are used as a substrate for the growth by the microorganisms [5]. Bacteria for the degradation were collected from MTCC as freeze-dried. The consortium of bacteria, *Pseudomonas* sp., *Micrococcus* sp., and *Bacillus* sp., were used in degradation [8]. The organism utilizes and degrades the hydrocarbon as sole source of energy. These organisms were added to the contaminated soil sample. Addition of nutrients helps them enhancing their rate of degradation (Figure No.1). The change in color showed the degradation rate of the organism. The medium showed heavy orange-green shade (Figure No.1a). After few weeks the color changed creamy (Figure No.2b) and then a layer was observed (Figure No.1c). The addition of carbon sources indicated that Ribose content increased the rate of degradation. The results indicate all three organisms in consortium were capable of utilizing and degrading more quantity of the hydrocarbon present in oil as nutrient source. The percent of degradation after 30 days of study was 90% under the assay condition [17, 18, 19]. These showed the consortium had higher degradation rate. There was an increase in cell number of the organism during the degradation process. The consortia proved to be a better degrader having degradable rate of 90% in 30 days. The optimization using different sources determined the complete degradation of hydrocarbons.

Effect of Carbon Sources on Hydrocarbon Degradation

Carbon source is among the various sources that are important for the production of energy in microorganism. These sources binds to the hydrocarbon and help in utilizing by the microorganism. Glucose, Lactose, Ribose, Sucrose, Maltose were used in the process. Ribose was found to be the most enhancing substrate in degradation of Kerosene, petrol, and diesel with a concentration of 0.505 ± 1.463, 0.154 ± 2.115, and 0.003 ± 0.135, respectively. However, Maltose and lactose showed less degradation of the hydrocarbon content. (Figure No.2) This showed that maltose and lactose were not utilized as carbon sources by the organism [11].

Effect of Nitrogen Sources on Hydrocarbon Degradation

Among various organic nitrogen sources, Peptone was found to have significant effect on the degradation of hydrocarbons of Kerosene, petrol, and diesel with a concentration of 0.004 ± 0.705, 0.580 ± 0.817 and 0.019 ± 0.660, respectively among the other nitrogen sources that were used. (Figure No. 3).

Effect of Metal Ions on Hydrocarbon Degradation

Among various metal ions, Magnesium chloride was found to have significant effect on the degradation of hydrocarbons of Kerosene, petrol, and diesel with a concentration of 0.227 ± 1.122, 0.219 ± 1.783 and 0.644 ± 0.553, respectively among the other metal ions that were used. (Figure No.4)

Effect of pH in the Hydrocarbon Degradation

The maximum degradation rate was obtained at pH 8, which showed the optimum pH for the degradation of hydrocarbon and the initial pH level for enzyme synthesized, although the enzyme was active in the pH range of 6-9. The effect of pH 8 of the medium on hydrocarbon degradation Kerosene, petrol, and diesel were 0.270 ± 1.212, 0.058 ± 0.799 and 0.486 ± 1.351 respectively. (Figure No.5)

Effect of Temperature in Hydrocarbon Degradation

The optimum temperature appears to be 40^0C, the initial temperature for the enzyme synthesized during the degradation process. The enzyme activity was stable at 25–40^0C with a residual activity greater than 60%. (Figure No.6) The temperature was found to influence the degradation rate.

Dry mass determination

The dry mass was determined using the initial value and final value of the soil before soxhalation. Hydrocarbon biodegradation was apparent at rates of less than 1µg of hydrocarbon per g (dry weight) of sediment per day.

Figure No.1a

Figure No.1b

Figure No.1c

Figure No.1: Microbial Degradation of Hydrocarbons

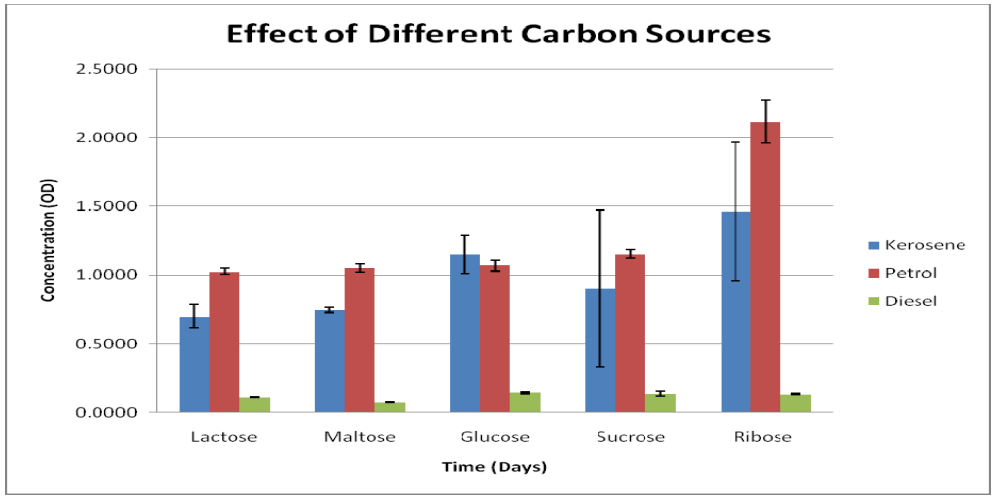

Figure No.2

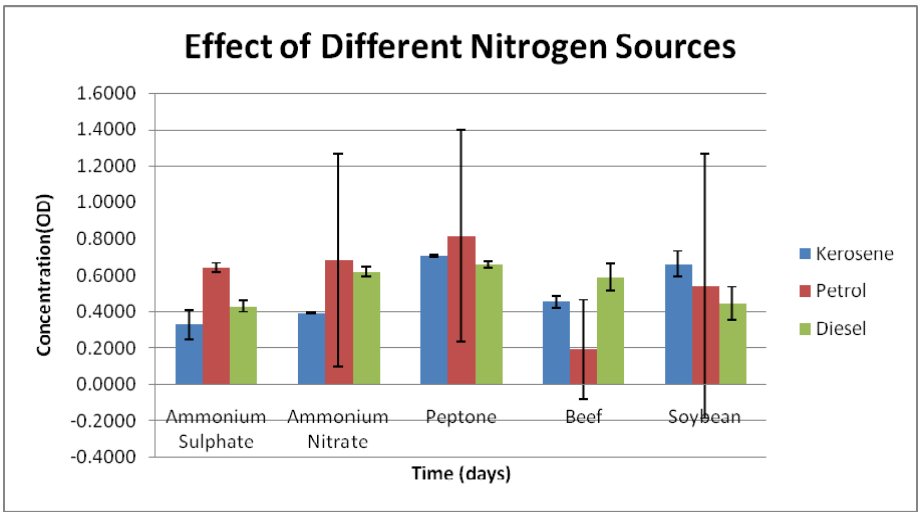

Figure No.3

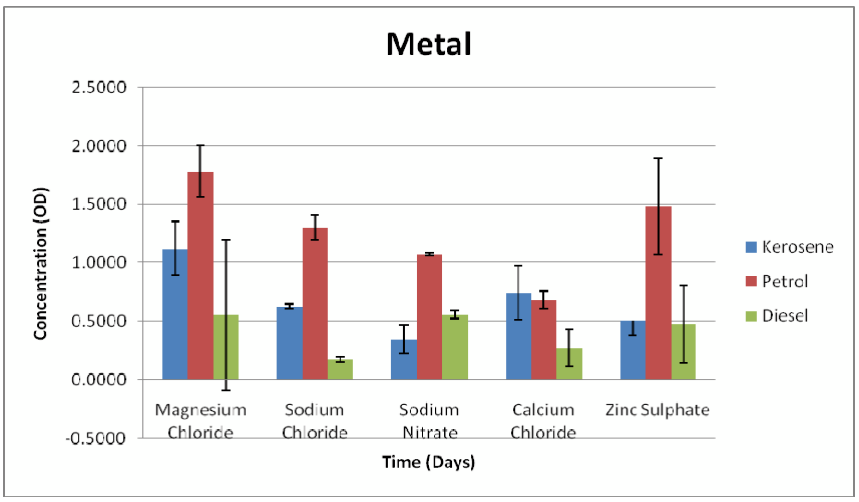

Figure No.4

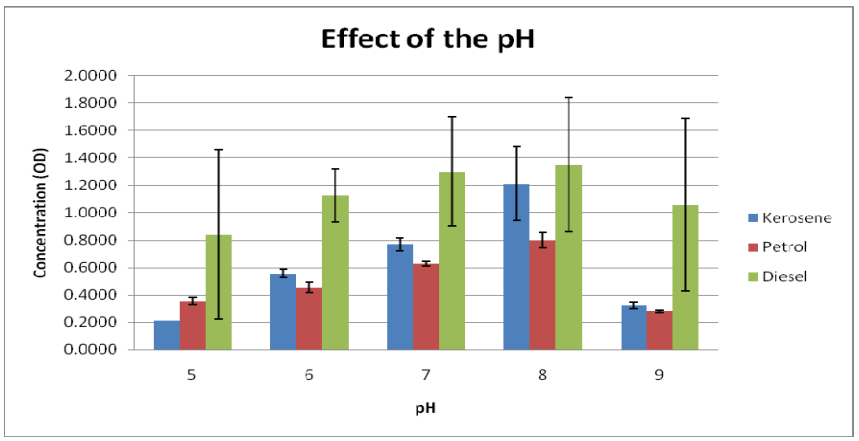

Figure No.5

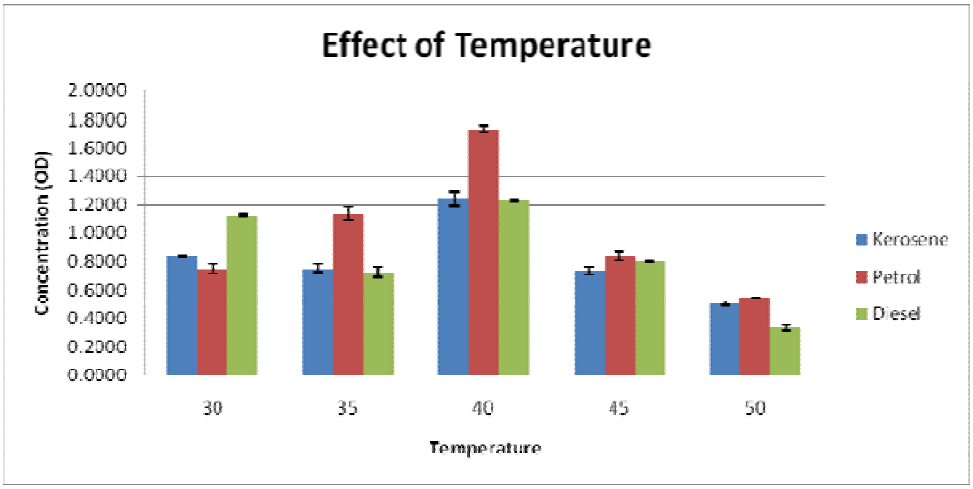

Figure No.6

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