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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF PETROLEUM HYDROCARBON DEGRADING INDIGENOUS BACTERIA FROM CONTAMINATED SITES OF VISAKHAPATNAM

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Abstract

The wide spread use of petroleum products leads to contamination of soil and aquatic environments, thereby poses a serious threat to all life forms counting humans. The ecology of hydrocarbon degradation by microbial populations in the natural environment is reviewed, emphasizing the physical, chemical, and biological factors that subsidize to the biodegradation of petroleum and individual hydrocarbons. Seventeen bacterial isolates able to grow on crude oil were isolated from various hydrocarbon-contaminated sites in Visakhapatnam. These samples were screened for bacterial oil degradation using 0.5% diesel in Bushnell-Hass Mineral Salt medium. The level of petroleum hydrocarbon degradation was determined by turbidometry and DCPIP methods at each 7 days interval. These organisms were studied to determine their biodegrading activities on hydrocarbons (diesel and petrol) as the sole carbon source using enrichment medium. Maximum utilization of hydrocarbons from crude oil was indicated by the total discoloration of DCPIP. Screening for bacteria utilizing crude oil as the sole source of carbon with 2, 6-dichlorophenol indophenol (DCPIP) as redox indicator was carried out for all the selected isolates. Based on their capability to degrade hydrocarbons, six isolates were further selected to prepare a consortium which showed maximum utilization of hydrocarbons indicated by the total discoloration of DCPIP in just 90 hours.

Introduction:-

Hydrocarbons are the world's most commonly used primary energy and fuel resources, due to the energy they produce. Apparently inevitable spillages, which follow during routine operations of crude oil production, refining, distribution and as a moment of acute accidents, have engendered continuous research interest in this field (Okoh, 2003). Oil spills have become a global problem in industrialized and developing countries. The amount of natural crude oil seepage was expected to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year (Kvenvolden, 2003).

These hydrocarbon contaminations are hazardous to the health of plants and are also carcinogenic, mutagenic and potent immuno-toxicants pretense a serious threat to human and animal health (Atlas, 1981; Zhou Crawford, 1995; Liebeg and Cutright, 1999, Ting and Hutan, 1999, Vasudevan and Rajaram, 2001). Petroleum products such as gasoline, kerosene, diesel/fuel oil and crude oil are a composite mixture of organic compounds basically of paraffinic, oleifinic and polycyclic aromatic hydrocarbons (Mittal and Singh, 2009; Singh and Lin, 2008; Vieira *et al*., 2007).

Presence of Polycyclic Aromatic Hydrocarbons (PAHs) in soil and water are a foremost problem as environmental contaminants and most of these PAHs are intractable in nature. PAHs mean a prospective risk to the marine animals as well as to the human health as many of them are carcinogenic.

Although potentially beneficial methods to improving substrate availability, chemical pre-treatment (Kornmuller and Wiesmann, 1999; Stehr *et al.*, 2001), or increasing mass/diffusion transfer rates at higher temperatures (Freitkenhauer *et al.*, 2003) may be adopted, these measures may add to the environments stress problems and treatment costs. Bioremediation functions mainly on biodegradation, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds and cell protein or conversion of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms. In addition, bioremediation technology is supposed to be non-invasive and moderately cost-effective.

Indigenous oil consuming microorganisms, which have the ability to degrade organic compounds play a significant role in the disappearance of oil from soil. This microbiological decontamination (bioremediation) of the oil-polluted soils is claimed to be a competent, economic and adaptable alternative to physiochemical treatments (Atlas 1991, Bartha, 1986).

This paper deals with sampling, chemical analysis, quantitative bacteriological analysis, isolation and screening of hydrocarbon degrading bacteria from crude oil, soil and effluent samples from the HPCL oil refinery, Visakhapatnam. It also deals with screening for quantitative and qualitative biodegradation potential of the pure cultures of selected bacterial isolates as well as their association by DCPIP method.

The strains were selected based on the criteria that they were able to perform decolorisation of DCPIP (utilization of hydrocarbon component (s) of the crude oil in a relatively short time). Screening of individual microbe/association for utilization/degradation of pollutants is an important task to progress the bioremediation processes [Bidoia, 2010]. 2, 6-dichlorophenol indophenol (DCPIP) based colorimetric assay can be used to study the degradation of hydrocarbons by microorganisms. In oxidized state the indicator is blue and its reduced state is colourless. The colour change is due to structural change in the molecule, in which the double bond between nitrogen and carbon permits to a single bond. The colour change is directly proportional to degradation of hydrocarbon compound (s) [Mariano, 2008; Varjani, 2013; Bidoia, 2010; Hanson, 1993]. Present study is aimed to investigate the employment of hydrocarbon contaminants by indigenous bacterial isolates individually and their association.

Materials and methods:-

Samples Collection:-

Microorganisms used in the degradation study were isolated from sludge samples and effluent samples collected from the HPCL oil refinery, Visakhapatnam.

Isolation of hydrocarbon-degrading bacteria:-

Hydrocarbon-degrading bacteria present in the soil and water samples were isolated in two ways: (a) by direct plating of dilutions of the samples on mineral salts agar containing crude oil as the only carbon and energy sources; and (b) by plating of enrichment cultures of the samples prepared in mineral salts broth, also containing crude oil as the only carbon and energy sources. Ten grams of prepared soil samples was suspended in 100 ml sterile physiological saline, by vortexing, before being diluted up to 10^4. The soil samples were prepared by filtering through sterile 1 mm mesh screen to remove gravels and plant debris. Aliquots (0.1 ml) of the soil dilutions were plated on the mineral salts agar containing an overlay of 0.5 ml sterile Burgan crude oil. The mineral salts medium composition was (gm/l): K_2HPO_4, 0.5; Na_2SO_4, 2.0; NH_4Cl, 1.0; $CaCl_2.7H_2O$, 0.15; $MgSO_4.7H_2O$, 0.02, agar 15; final pH, 7.2. The water samples were also diluted by transfer of 10 ml into 90 ml physiological saline, and diluted up to 10^{-4}, before plating 0.1 ml aliquots on the mineral salts agar containing crude oil. Enrichment cultures were prepared by the addition of 10 g of each soil sample, or 10ml of water sample into 100ml mineral salts broth contained in screw-capped 250ml Erlenmeyer flasks, containing 1ml of the sterile crude oil. The cultures were incubated at 30^0_C in Gallenkamp Orbital Incubator (Weiss–Gallenkamp, Loughborough, U.K), agitated at 200 rev min^{-1} before 0.1 ml of the enriched culture was transferred into fresh sterile mineral broth. After such four successive weekly transfers, 0.1 ml aliquots were plated on nutrient agar. The mineral salts and nutrient agar cultures were incubated at 28^0C for 3 days, respectively, for adequate colony development. Distinct colonies were picked and purified by restreaking two times on nutrient agar before storage as slant cultures at 4^0C.

Determination of Bacterial Biodegradative:-

Activity by Turbidometry:-

Turbidometry is to regulate the bacterial growth by utilizing the hydrocarbons (1% petrol and diesel given as carbon source in MSM broth. This shows whether the bacterium possess the degrading activity of hydrocarbons like phenol,

petrol and diesel. The degrading activities of each isolates were attained by using Mineral salt broth (MSB) in which 1% of each hydrocarbon (petrol and diesel) was added and incubated at room temperature for 15 days. The growth of the bacterium was measured by taking the O.D readings at 595nm from 0hrs - 15 days at regular intervals of 2 days against mineral salt medium as blank.

Screening of hydrocarbon-degrading bacteria:-

Screening of potential hydrocarbon-degrading bacteria was carried out by adapted Hanson *et al.,* (1993); Bidoia *et al*., (2010) method using DCPIP as redox indicator. This technique was also employed in other studies (Afuwale and Modi, 2012; Joshi and Pandey, 2011; Mariano *et al*., 2008). During the microbial oxidation of hydrocarbons, electrons are transferred to electron acceptors DCPIP to the culture medium, and it is possible to ascertain the ability of the microorganism to utilize hydrocarbons by detecting the colour change from blue (oxidized) to colorless (reduced), which is monitored at 600 nm wavelength. Inoculum was prepared by relocating cultures from Nutrient agar slants into BH medium for 24 h at 37±2ºC at 180 rpm. Cultures were then inoculated into tubes along with DCPIP indicator (0.5% w/v) and the selected crude oil (3%, v/v) for spectrophotometric analysis. All the tubes were incubated at room temperature for 144 h. The absorbance of all the assays was measured by Visible spectrophotometer. Data was collected at regular time interval of 24 h till 96 h.

Bacterial identification:-

The morphological characterization of each isolate was first performed, including color and size. Gram stain test was performed for each isolate (Fig 3, 4).

Biochemical characterization of selected microorganisms:-

Biochemical tests; urease production, starch hydrolysis, carbohydrate fermentation (lactose and sucrose), catalase were performed with isolated hydrocarbon consuming bacteria (Table 1).

Starch hydrolysis test:-

Inoculated a starch plate with the organism to be tested. Incubated at for at least 48 hours. Plates were swamped with iodine solution and observed results. Blue colour indicates no hydrolysis, while a clear zone indicates hydrolysis. The plates were detected for starch hydrolysis as when iodine added, a colour change is blue but area which shows positive result.

Catalase test:-

A few drops of bacterial broth culture were placed on cavity slide. Same amount of hydrogen peroxide were released on plate. The plate was observed for bubble formation.

Results and discussion:-

Isolation and Identification of the Bacteria:-

The bacteria were isolated from three different types of samples on nutrient agar medium. Further the samples were screened for the presence of hydrocarbon degrading bacteria on mineral salt medium with 1% of the hydrocarbons as the only carbon source namely petrol and diesel separately. Hydrocarbons are needed as a carbon source but it can be toxic to microorganisms due to the solvent effects of diesel and petrol that could terminate bacterial cell membrane. Many biodegradation studies were reported on diesel are carried out using lesser diesel concentrations ranging from 0.5 to 1.5%. But M.Y Shukor *et al*., (2009) reported degradation of diesel by microorganisms at 3.5% and 6% diesel by Kwapisz *et al.,* (2008). It has been found that degradation is generally unfavorable at concentrations higher than 1 or 1.5% [Bicca, 1999; Espeche, 1994]. Number of colonies on mineral salt medium is lower when compared to the mother plate without hydrocarbons (Fig 1, 2). This result showed that the bacteria grown on enriched medium were able to degrade the hydrocarbon source.

Hydrocarbons by Turbidometry:-

The O.D readings based on the turbidity of MSM broth at regular intervals of 1 day give the degrading activity on hydrocarbons by bacteria. The graphs based on the O.D readings at various time intervals of incubation period on the degrading activity of the oil-degrading bacteria are also showed in the Fig 5. Our results showed that all the organisms utilized all the hydrocarbon substrates (petrol and diesel) when supplied as the only source of carbon and energy although, the level of utilization differs from one microbe to another (due to differences in their growth) and from one hydrocarbon substrate to the other, due to the obvious differences in their molecular sizes. These degrading capabilities on different hydrocarbons discovered that the microorganisms isolated from the soil and water samples

were able to degrade hydrocarbons. The cells were able to increase within the days of study, indicating that they were able to degrade and consume the oil for their growth and development, hence the concomitant increase in the concentration of the broth (turbidity). This gradual increase in the concentration of the broth shows bacterial growth hence degradation of hydrocarbons, mostly between days 2 and 3 gradual decline in the concentration of the broth suggests decrease in the bacterial population and that the hydrocarbon has been degraded, mostly between days 5 and 7.

Screening of hydrocarbon utilizing bacteria by DCPIP method:-

Following isolation and improvement of all the seventeen isolates were screened for their efficiency of crude oil utilization/degradation (as a sole carbon source) using 2, 6- dichlorophenolindiphenol (DCPIP). Colour change of DCPIP was observed visually till 144 h. Based on the time for decolouration of DCPIP Six isolates were selected for further studies. Six isolates which decolorized DCPIP in the shortest time (about 120 h) were chosen for preparing the association. Both the isolates and association were further studied for decolorisation of DCPIP spectrophotometrically by measuring absorbance at 600 nm, periodically (Bidoia *et al*., 2010)(Fig 6)

Microbial bioremediation as well as bioaugmentation are commonly used techniques for treating hydrocarbon pollution in both terrestrial and aquatic ecosystems. Indigenous hydrocarbon degrading microorganisms play a significant role in this process. Research for regulating *in situ* degradation process is required for a successful full-scale operation (Bidoia *et al*., 2010). The physico-chemical properties of oil-spill contaminated sites are important for successful bioremediation process. These factors have direct effect on the type, number and metabolic activities of the microflora of any ecosystem (Adebusoye *et al*., 2008).

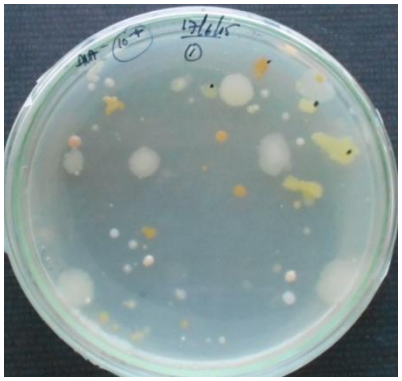

Figure. 1: Isolation plate

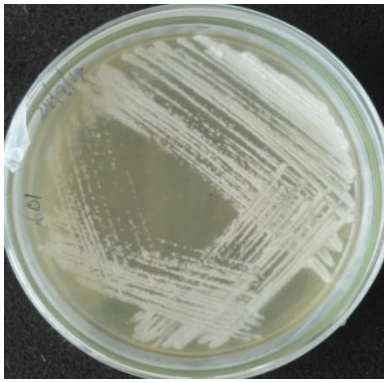

Figure. 2: Pure culture plate

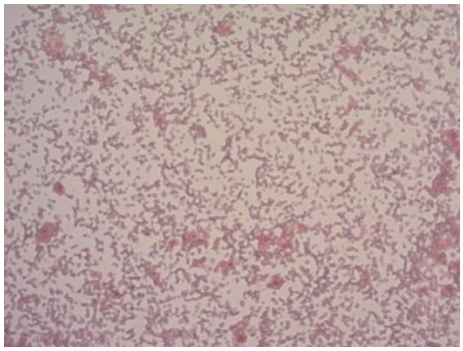

Figure. 3: Grams staining

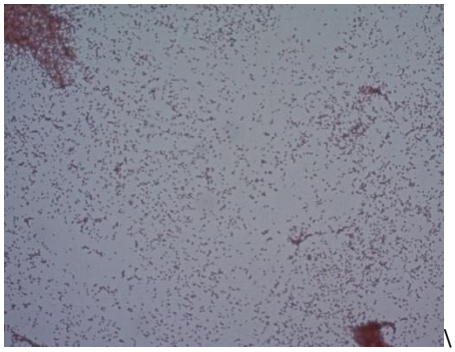

Figure. 4: Grams staining

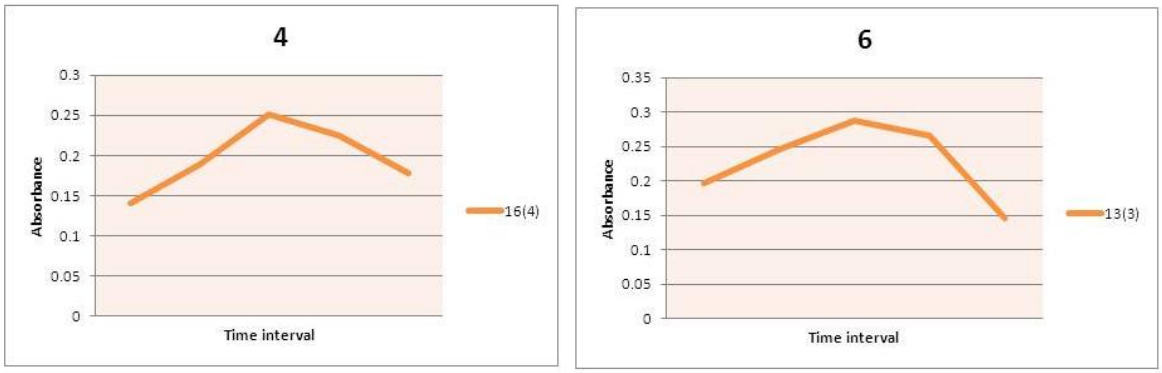

Figure. 5: Graphs showing the Turbidity of the selected isolates

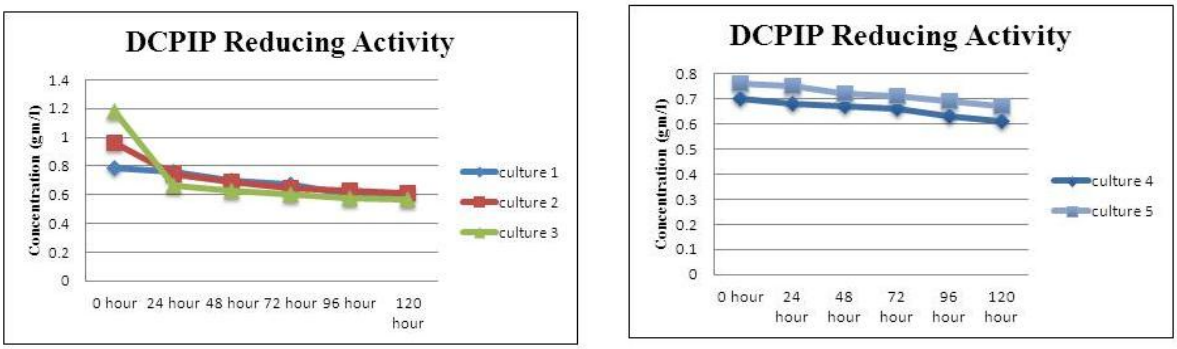

Figure. 6: Graphs showing the degradation of DCPIP.

Table. I: Biochemical characterization for selected isolates

Feature	AUBT – 001	AUBT – 002	AUBT – 003	AUBT – 004	AUBT – 005	AUBT - 006
Gram Stain	+ ve	- ve	+ ve	+ ve	- ve	+ ve
Shape	Rod	Round	Round	Round	Rod	Round
Catalase	+ ve	+ ve	- ve	+ ve	- ve	+ ve
Starch Hydrolysis	+ ve	+ ve	- ve	- ve	- ve	+ ve
Indole	- ve	+ ve	+ ve	- ve	+ ve	- ve
MR-VP	+ ve	- ve	- ve	- ve	- ve	+ ve
Oxidase	+ ve	- ve	- ve	+ ve	+ ve	+ ve
Glucose	- ve	+ ve	- ve	+ ve	+ ve	+ ve
Coagulase	+ ve	+ ve	- ve	- ve	- ve	
Mannitol	+ ve	- ve	- ve	- ve	- ve	+ ve
Citrate	- ve	- ve	- ve	- ve	- ve	- ve

Conclusion:-

Bioremediation is one of the most rapidly rising areas of environmental biotechnology, which has been used for the cleaning up of pollutants. This is because of its low costs and its public suitability. Degradation of hydrocarbons by environmental micro florae includes microorganisms having particular metabolic capacities. In polluted environments, particular microorganisms are abundant because of the variation of the micro florae to pollutant. It is apparent from this study that, hydrocarbon degrading organisms are ubiquitous in environment and they can be isolated from hydrocarbon polluted sites and waste water. It has also been shown that six bacterial strains isolated from contaminated soil can be good petrol and diesel degraders. This study can focus on more cost effective applications of native bacterial strains for petrol and diesel degradation at large scale in industries, where it pose an alarming problem due to its detrimental health effects on different organisms and human beings. The degrading capacity demonstrated by the microorganisms is a clear indication that they retain a gene that is used in hydrocarbon degradation.

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