"ENZYMATIC STUDIES AND MINERALIZATION POTENTIAL OF SOME BACTERIAL ISOLATES FROM SOIL CONTAMINATED WITH PETROLEUM HYDROCARBON RESIDUES"

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Abstract : Petroleum hydrocarbon mineralization is of central importance in ecosystem functioning. The mineralization process is transformation of organic compounds into inorganic form by the soil organisms. Microbial population indigenous to petroleum contaminated soil are suitable for such mineralization process that aid bioremediation. The petroleum hydrocarbon utilizing bacterial isolates from the experimental soil samples include *Pseudomonas* spp, *Staphylococcus* spp, *Bacillus* spp, and *Corynebacterium* spp. These microbial isolates and their consortium of mixed cultures of two, three and four cultures exhibiting mineralization of petroleum residue (1% diesel) was investigated for a period of 60 days. Among the four isolates, ,highest value was obtained for the rate of petroleum residue degradation was with *Pseudomonas* spp (78%), which also showed highest emulsification rate (60%). The CO_2 evolution of 55 gm kg^{-1}, cell biomass (175 CFU) and 50 % rate of emulsification observed with Consortium 1, a mixed culture of *Pseudomonas* spp & *Staphylococcus* spp.;as compared to the other ten consortium studied. Enzyme dehydrogenase activity was observed highest in the inoculum of *Staphylococcus* spp (84.1UM/min/ml) indicating the potential to degrade petroleum hydrocarbon. The GCMS data also supports our finding that consortium of *Pseudomonas* spp & *Staphylococcus* spp are potent to mineralize diesel up to the range C_6 to C_9 from C_{19}-C_{44} found in the control samples.

Index Terms **– Hydrocarbon Residue, Mineralization, Enzyme, Microbial Consortium**

INTRODUCTION

Nature has been a potential source of oil degrading agent for years. Recently, attention has been given to oil pollution remediation and microbial enhancing oil recovery processes (Shahaby et. al., 2015). Petrol and petroleum residues are a risk for human and animal health, since many of these contaminants have demonstrated to be toxic and carcinogenic. Petroleum contaminated soil causes organic pollution of local ground water. Bioremediation technique is sought as an alternative methods for treatment of contaminated soil. These techniques are economically and politically attractive and have shown promising results in the treatment of soil contaminated with organic compounds, particularly with petroleum hydrocarbons (Yerushalmi et. al., 2003). Individual microorganisms are capable of degrading only a limited number of crude oil, hence bioremediation extensively depends on the presence of metabolically diverse microbial communities (Vinothini et. al., 2015). The exploitation of the metabolic versatility of microorganisms is advantageous in bioremediation but the actual number of petroleum hydrocarbon degraders in a mixed culture may only represent 5-10% of the microbial community (Sawadogo et. al., 2014). There is an extensive literature on degradation and mineralization of hydrocarbons by microorganisms. Degradation is interpreted as the disappearance of original hydrocarbon by process other than volatility. In contrast, mineralization designates complete recycling to inorganic compounds and is meaningful term in the relation to pollution problems (Atlas and Bartha., 1971). In a recent study, the capability of native bacterial population to mineralize petroleum hydrocarbons in refinery sludge was studied by Ajao et. al.,

(2014). Mineralization is a combined activities of microbial consortia that does not produce intermediates pollutants in the environment, but the final products are CO_2 and H_2O (Freijer et. al., 1996). Thus to understand how microorganisms may be exploited so as to reduce and shorten the start-up times for the treatment of soil contaminated by organic compound, therefore in the present study the important bacteria actively involved in the degradation of petroleum residue were isolated and screened from soil contaminated by petroleum residues and investigated their potential to mineralize and degrade diesel oil.

Materials and methods

The adjoining area of a petrol pump in the Devrukh village, District- Ratnagiri located in the suburban region of Konkan, Maharashtra state (India) was selected as the sampling site. Soil samples contaminated with petroleum residues from different locations ranging from close proximity to the fuel tank extending to the radius of 10m from the fuel tank within the study area were collected for the study. The samples were labelled, sealed separately in a sterile bag and stored in the laboratory for further use (Gulati et. al., 2017).

Isolation and Identification of Petroleum Degraders

The isolation of petroleum degrading bacteria species was done by enrichment of Bushnell-Hass medium (BH) with 1% diesel as petroleum residues(Madhavi et. al., 2014). Ten gm of oil contaminated soil samples collected from each sampling site was added to 250-ml Erlenmeyer flask containing 50 ml of BH medium and incubated at 37ºC for 10 days. After 10 days, pure culture was isolated by plating out BH broth on BH agar and incubated at 37ºC for 24 hrs. Isolates obtained were inoculated on BH medium enriched with 1% Diesel as a sole carbon source (bought from same petrol pump station) and incubated at 37ºC for 24 hrs (Panda et. al., 2013). The isolates were subjected to biochemical and physiological characterization for identification to the generic level by following the taxonomic scheme of Bergey's Manual of Systematic Bacteriology (Kumari et. al., 2013).

Mineralization potential of isolated microorganisms

Mineralization potential of bacterial isolates and their consortium containing mixed culture of two, three and four isolates were evaluated. 100 ml of BH broth medium supplemented with 1 % Diesel oil as a sole source of carbon was transferred to 250 ml of Erlenmeyer flasks (under sterilized condition), inoculated with each bacterial isolates and consortia in separate flasks along with respective control (no diesel). For studying emulsification and degradation rate of petroleum residues control was maintained containing only diesel oil (no inoculation). All flasks were incubated in shaker orbital incubator at 37 ° C, 125 rpm for 0days, 7days, 15 days, 30 days and 45 days and 60 days. After the respective days of incubation the mineralization potential of each isolates and their consortium were analyzed for CO_2 production by titrimetric method (Doran and Zander, 2012), emulsification (Ramos et. al., 2010), pH and temperature of growth medium, cell biomass (Vinothini et. al., 2015), dehydrogenase enzyme activities spectrophotometrically (Yan et. al., 2014) and diesel degradation by Gravimetric assay (Shahaby et. al., 2015). All these experiments were performed in triplicates; standard error was calculated by Microsoft XL 2010.

After the 60 days of incubation period, 5ml of the each culture was extracted with 20ml of n-hexane as solvent using separating funnels to remove cellular material. The residues were transferred to tarred vials and the volume of each extract was adjusted to 100ml further by adding n-hexane and 1 % diesel oil (without inoculation) was treated as control. The vials were maintained at 4°C for the Gas chromatographic-Mass spectrometry analysis at SAIF, IIT Mumbai (Garima et. al., 2016).

RESULTS AND DISCUSSION

The isolates that degrade petroleum residues in the soil from the Devrukh petrol pump station were identified based on colony morphology, biochemical and physiological characteristics of the bacteria as per Bergey's Manual of Systematic Bacteriology. The isolated colonies were identified as *Pseudomonas spp, Staphylococcus spp, Bacillus spp, & Corynebacterium spp,* (Table No 1). Individual bacterial isolate has limited capacity to degrade crude oil, therefore the consortium of mixed culture with two, three and four isolates Viz; *Pseudomonas spp - Staphylococcus spp* (Consortium 1), *Bacillus spp - Corynebacterium spp* (Consortium 2), *Pseudomonas spp - Bacillus spp* (Consortium 3), *Pseudomonas spp - Corynebacterium spp* (Consortium 4), *Staphylococcus spp - Bacillus spp* (Consortium 5), *Staphylococcus spp - Corynebacterium spp* (Consortium 6), *Pseudomonas spp - Staphylococcus spp - Bacillus spp* (Consortium 7), *Pseudomonas spp - Staphylococcus spp -Corynebacterium spp* (Consortium 8), *Bacillus spp - Staphylococcus spp - Corynebacterium spp* (Consortium 9), *Pseudomonas spp - Bacillus spp - Corynebacterium spp* (Consortium 10), *Pseudomonas spp - Staphylococcus spp - Bacillus spp - Corynebacterium spp* (Consortium 11) were studied.

Table 1: Physiological and Biochemical Characteristics of bacterial isolates from petroleum hydrocarbon contaminated soil

	Bacterial Isolates From Petroleum Hydrocarbon Contaminated Soil			
	Pseudomonas Spp	***Staphylococcus Spp***	***Bacillus Spp***	***Corynebacterium Spp***
Gram Staining	-Ve	+Ve	+Ve	+ Ve
Shape	Rods	Cocci	Rod	Rods
Motility	Motile	Nonmotile	Motile	Nonmotile
Cultural / Morphological	Non Capsulated, Non Sporing, Flagellated	Flagellated,,Nonsporing, Noncapsulated Nonmotile	Transparent Non Capsulate Flagellated Spore Forming	Non Capsulated, Non Sporing, Flagella
Lactose	-	+	+	-
Glucose	-	+	**A** +	-
Sucrose	-	+	+	-
Mannitol	+	+		
Fructose			+	
H2S	-	-	-	-
Nitrate	+	+	+	+
Indol	-	-	-	-
Mr	-	+	-	-
Vp	-	+	-	-
Citrate	+	+	+	+
Urease	-	-	-	-
Catalase	+	+	+	+
Coagulase		+	-	+
Bacitracin		-		
Oxidase	+	-	Variable	+
Gelatine Hydrolysis	+	+	+	
Starch Hydrolase	-	+	+	-
Lipase	+	-	+	+
Tsi	-	-	-	-

The degradation pattern of the isolates from the experimental site after 10 days of selective enrichment in the 1 % diesel revealed the occurrence of hydrocarbon degrading bacteria in the soil contaminated by petroleum residues. The potential isolates, *Pseudomonas spp, Staphylococcus spp, Bacillus spp and Corynebacterium spp* demonstrated the ability to survive and proliferate in the presence of hydrocarbons and used diesel as sole source of energy and carbon. All these indigenous bacterial isolates seemed to have acclimatized to degrade crude oil (diesel). When the rate of degradation for each organism was studied(after 60 days of incubation), *Pseudomonas spp* demonstrated highest rate of degradation (74%) followed by *Staphylococcus spp* (72 %), *Corynebacterium spp* (45%) and *Bacillus spp* (36.4%). Thus from the results *Pseudomonas spp* can be treated as best isolate to degrade diesel. Similarly, when the rate of diesel degradation by bacterial consortium were studied (**Fig.1**), it revealed that among the 11 consortium, the mixed culture of two isolates *Pseudomonas spp - Staphylococcus spp* (Consortium 1) showed 78% and consortium of all four bacterial isolates *Pseudomonas spp - Staphylococcus spp - Bacillus spp - Corynebacterium spp* (Consortium 11) with 67% degradative ability. Therefore *Pseudomonas spp and Staphylococcus spp* can be treated as potent diesel degraders. Whereas consortium of three bacterial mixed culture *Pseudomonas spp - Staphylococcus spp- Corynebacterium spp* (Consortium 8) recorded with only 40 % rate of diesel degradation among others. Other consortium of mixed culture showed degradation ability, though very low to be treated as potent microbial mixed culture suitable for bioremediation.

The results of this investigation have shown the occurrence of certain potential petroleum residues degrading microorganisms in the experimental site of polluted environment. A total of four bacterial isolates ,identified to the genus level as. *Pseudomonas, Staphylococcus, Bacillus and Corynebacterium* can be treated as having the potential to degrade the petroleum residues. Ebacota et. al., (2017) and Mittal & Singh, (2009) have reported same bacterial isolates in addition to few other isolates in the soil contaminated by petroleum. Majority of the bacterial population isolated from the soil samples were Gram positive. Ability of isolates to survive in 1% of diesel during 10 days of enrichment technique is important as it seemed to have led to the synthesis of enzymes involved in diesel metabolism or changes in the genetic capacity of microbial species to maintain their ability to degrade crude oil. Mineralization is the decomposition of the chemical compounds in organic matter, by which the

nutrients in those compounds are released in soluble inorganic forms (Hossain et.al., 2017). In the effective bioremediation process, mineralization of petroleum residues can be evaluated by assessing the presence of carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms (Das N. and Chandan P., 2011). The rate of Mineralization is assessed by the evolution of inorganic CO_2 in the medium as well as the fate of change of pH and temperature of the medium showed that *Pseudomonas spp and Staphylococcus spp* (Consortium 1) exhibited highest CO_2 evolution i.e.55 $gmkg^{-1}$ (5.48 % more than control), followed by individual isolate *Pseudomonas spp* evolved 50 $gmkg^{-1}CO_2$ (4.97% more than control). Where as mixed culture of all four bacterial isolates *Pseudomonas spp - staphylococcus spp - Bacillus spp - Corynebacterium spp* (Consortium 11) and consortium of three bacterial mixed culture (Consortium 8) *Pseudomonas spp - Staphylococcus spp- Corynebacterium spp* was able to produce 40 $gmkg^{-1}$ (3.98 % more than control)and 39 $gmkg^{-1}$ (3.87 % more than control) CO_2 respectively. In case of other consortium the rate of CO_2 production was lesser as compared to Consortium 1, 8 and 11 (**Fig.2**) hence can not be considered as suitable for the mineralization of hydrocarbon in efficient bioremediation process. CO_2 evolution has been used as indicator of bacterial respiration rate, a product of the bioremediation process (Kao et. al., 2001). In the present study, mineralization of petroleum residues as measured by CO_2 evolution was proportional to the rate of degradation, emulsification, population of microorganisms. The temperature of the test medium was observed to have slightly increased within 60 days of incubation period. This might be due to the production CO_2. The results revealed that the isolates and their consortium was able to mineralize petroleum residues.

The success of biodegradation of petroleum residues highly depends on presence of microorganisms with appropriate metabolic capabilities and adaptability to survive in the growth medium. Microbial population is enumerated by counting cell biomass in the growth medium. The study results revealed that cell biomass of all the bacterial isolates *Pseudomonas spp, Staphylococcus spp, Bacillus spp, Corynebacterium spp* and their consortium tend to increase proportionately at each time interval. However, mixture of two isolates i.e. *Pseudomonas* spp and *Staphylococcus* spp (Consortium 1) recorded highest cell biomass (175 CFU), followed by mixed culture of all four isolates (consortium 11) exhibited 160 CFU where mixed culture of three i.e. *Pseudomonas spp, Staphylococcus spp and Bacillus spp* (consortium 7) showed 159 CFU bacterial mixed population (**Fig.3**). In the study presented, cell biomass of the all the isolates found to be increased with time. Many researchers reported increase of cell biomass while degrading the organic compounds due to higher extent of adaptability and change in metabolic pathway.

During the 60 days of incubation period, metabolic activities by the microorganisms in the medium influenced the pH & it was in the range 5 to 6.5 ,thus contributing to the pH fluctuation from acidic to alkaline. The temperature of the medium was also recorded to have increased slightly at each time interval (**Fig.4**). Similar results are reported by many researchers that increase in temperature during mineralization was indication of CO_2 production.The reduction in pH of the medium in the experimental flasks within the incubation period further confirmed chemical changes of the hydrocarbon substrates which must have been precipitated by microbial enzymes. It has been reported that microbial degradation of hydrocarbon often leads to production of organic acids and other metabolic products. Thus, the acids probably produced accounted for the reduction in the pH levels. This is important in view of the microbial survival and adaptation in terms of suppressing effect on the synthesis of enzymes involved in crude oil metabolism or by changes in the genetic capacity of microbial species to maintain their ability to degrade crude oil (Ajao et. al., 2014).

Also the result of emulsification revealed that, individual *Pseudomonas spp* showed highest emulsification rate i.e. 60% followed by mixed culture of *Pseudomonas* spp & *Staphylococcus spp* (consortium 1) 50 % and mixed culture of all four isolates (Consortium 11) 47 % when supplemented with 1% diesel (**Fig.5**), indicating that these microorganisms are capable of utilizing up to 1% diesel. Onuoha et.al., 2011 too have reported similar findings of rate of emulsification while studying lubricating oil in Nigeria. Dehydrogenase activities for non diesel induced and diesel induced bacterial isolates (**Fig.6**), showed highest dehydrogenase enzyme activity by *Staphylococcus spp* (84.1 UM/min/ml) followed by *Pseudomonas spp* (65.5 UM/min/ml). Whereas in other isolates the activity was comparatively lower *Bacillus spp* (37.5 UM/min/ml) and *Corynebacterium spp* (31.3 UM/min/ml). Moreover, the activity of dehydrogenase enzyme was much higher in the isolates induced by 1 % diesel than in the noninduced ones, indicating that the dehydrogenase of the isolates may be inductively expressed in the presence of 1% diesel. The role of dehydrogenases consists in the biological oxidation of organic matter in the soil by hydrogen transfer from the organic substrate to inorganic acceptors (Zhang et. al. 2010). Presence of dehydrogenase activity in the test culture filtrate after 60 days of incubation can be attributed to the inducible enzymes involved in biodegradation due to the presence of utilizable hydrocarbon substrates. Dadrasnia A. & Agamuthu P. (2013) also monitored dehydrogenase activity and CO_2 evolution at the time of biodegradation of diesel fuel.

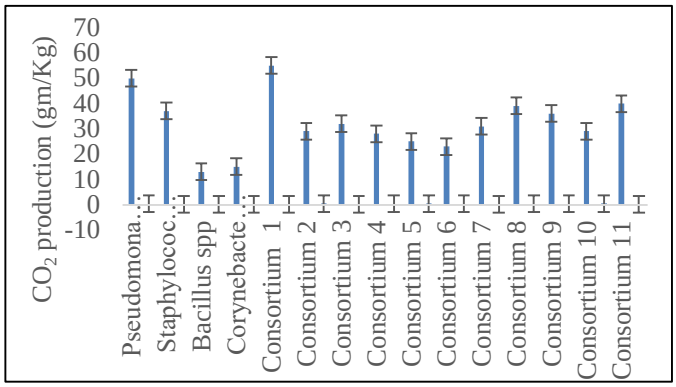

Figure1 :Rate of diesel degradation by bacterial isolates , their eleven consortium and control

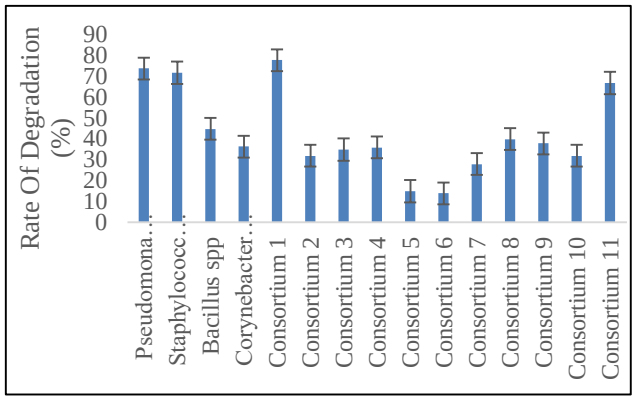

Figure2: Rate of CO_2 production by bacterial isolates ,their eleven consortium and control

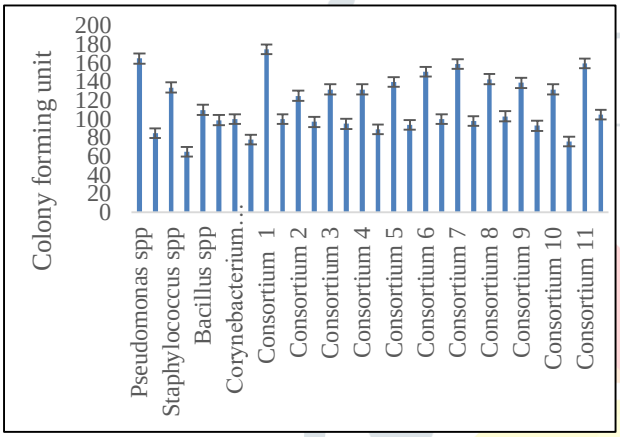

Figure3: Cell biomass (CFU) of bacterial isolates, eleven their consortium and control

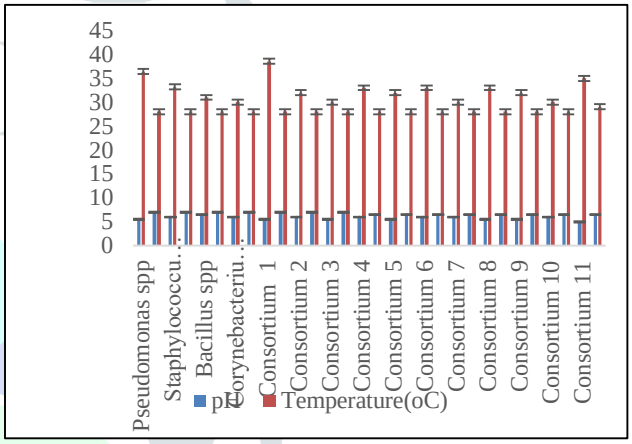

Figure 4:pH and Temperature of growth medium inoculated with bacterial bacterial isolates ,their consortium

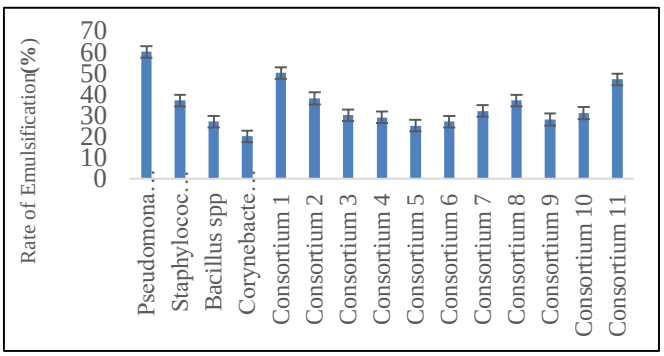

Figure5: Emulsification ability of bacterial isolates,their eleven consortium

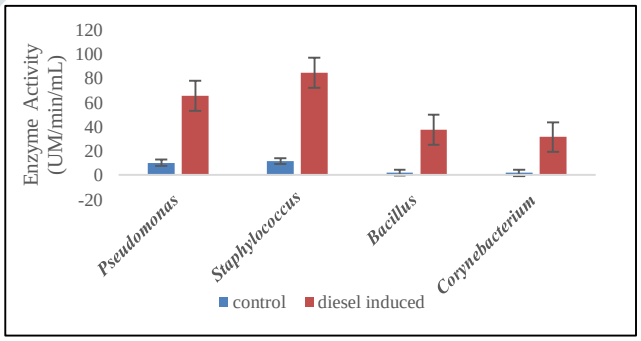

Figure6: Dehydrogenase enzyme activity by oil induced isolates and control

Gas Chromatography mass spectrometry resolved and elucidated various hydrocarbon component of diesel oil. GC peaks were found to vary between C_{19} to C_{44} in the control sample (**Fig.7a**) while the peaks obtained after degradation of diesel by *Pseudomonas spp* (**Fig.7b**) and *Staphylococcus spp* (**Fig.7c**) were in the range C_6 to C_{10}. Similarly degradation by consortium1 (**Fig.7d**) was observed to degrade the diesel up to the range C_6 to C_9. CO_2 production, increase of temperature and degradation rate was positively correlated which was proportional to the GC data. Gas chromatography and Mass spectrometry elucidated the degradation pattern of diesel. Before the degradation, the main peaks were C_{19} to C_{44}, *Pseudomonas spp* degraded up to C_6 to C_{10} within 60 days at 74 % in culture broth while *Staphylococcus spp* degraded C_6 to C_{10} at 72 % and their consortium degraded C_6 to C_9 within the same period resulting to 78% in culture broth. Peak reduction is a strong indicator for degradation, Namaji et. al., 2008 reported 66 % degradation of aliphatic compounds during 60 day. The higher biodegradation rate and microbial activity observed in the mixed culture may be related to shift in importance of one metabolic pathway over another possibly as a result of microbial synergism. The assumption that, more than one hydrocarbon compound degradation pathway exists in different microbial species and therefore, it is possible that individual bacteria able to degrade more than one aromatic substrate will have more than one pathway for their metabolism.In spite, of the fact that individual bacteria was isolated after 10 days enrichment in the presence of hydrocarbons as sole source of energy and carbon, ability to survive and mineralize crude oil therefore is significantly limited. This might be explained by the occurrence of mutualistic relationship in the enrichment process in this study.

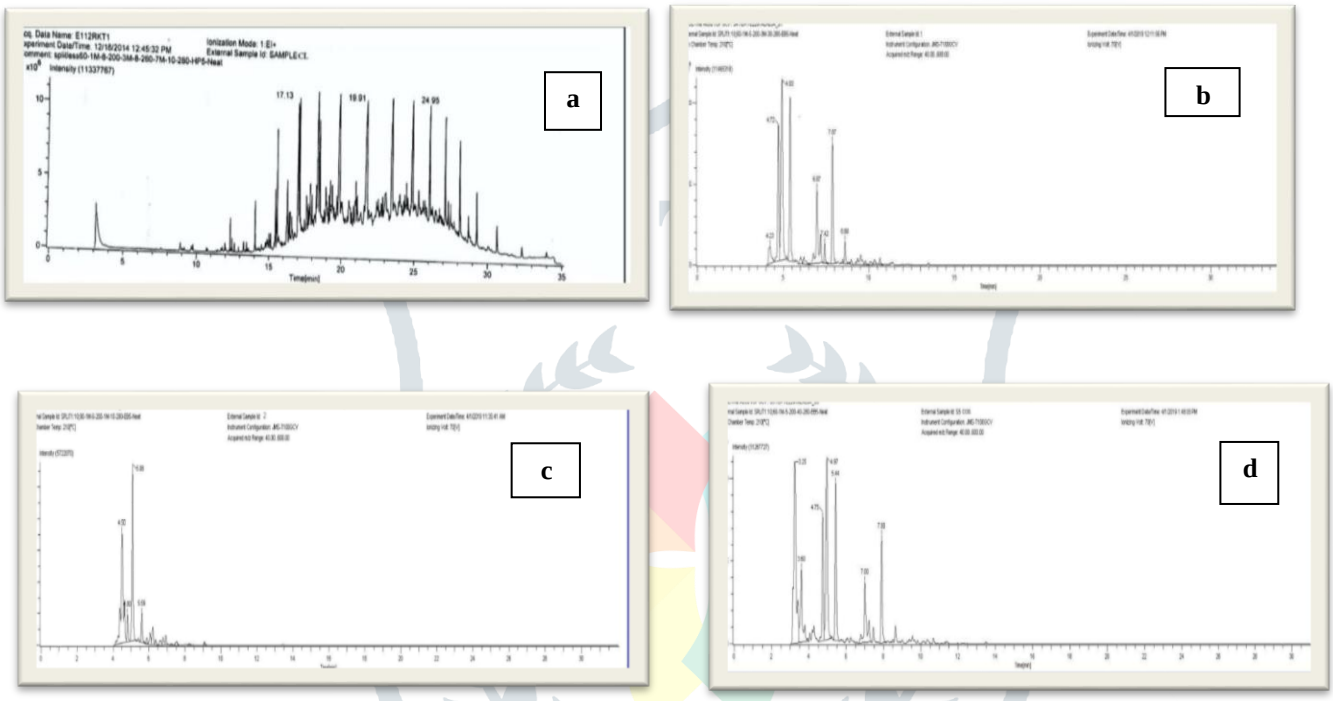

Figure 7. Gas Chromatographic Fingerprints of Diesel recovered from **a**: control (without bacterial isolates), **b**: Inoculated with *Pseudomonas spp* , **c** : Inoculated with *Staphylococcus spp*, **d**: Inoculated with mixed Culture of *Pseudomonas spp* and *Staphylococcus spp* (Consortium 1)

Conclusion

Bioremediation is surely one of the best way to combat petroleum residue soil pollution.The isolates *Pseudomonas spp, Staphylococcus spp, Bacillus spp,* & *Corynebacterium spp* appear to have the potential to contribute towards the degradation of the petroleum hydrocarbons in the soil. Evaluation of the biodegradation potential of microorganisms and their consortium in soil contaminated by petroleum residue was possible by studying CO_2 production, change in pH & temperature, dehydrogenase activity, rate of emulsification which are the biological indicators for assessing the mineralization of petroleum residue, with greater level than control soils. The protocols developed in this study may help to monitor natural attenuation and select bioremediation strategies to be applied for petroleum contaminated soils. The bacterial isolates and their consortium showed the potential to degrade the petroleum hydrocarbon residues in the medium (1% diesel) effectively. Further experimental work in the field may be of help to achieve bioremediation of contaminated soil.

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