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Diesel Oil Utilization Efficiency of Selective Bacterial Isolates from Automobile Workshop and Thesjaswini River of Kerala

Usharani, K.*, Sreejina, K.,Sruthi, T. and Vineeth, T.

Department of Environmental Science, School of Energy, Environment and Earth Sciences, Central University of Kerala, India

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ABSTRACT: Bioremoval and bioreduction activities of hydrocarbon (diesel) isolation from environmental samples were studied by the activity of biosurfactant production, and calculating emulsification index, gravimetric, and FTIR analysis along with the estimation of bacterial biomass. Sample from soil near petrol, diesel pumps and water sample from Thesjaswini River near Padannakad, Kasaragod, Kerala, India, were used to screen the potential diesel oil utilizing bacteria. Among the bacterial isolates (*Staphylococcus, Bacillus* and *Corynebacterium* strains), *Staphylococcus* sp was the potent degraders of diesel oil. *Staphylococcus* strain was observed to be maximum diesel oil utilizing ability (73% emulsification index) and change in the functional groups of the compound (FTIR analysis). The strain showed optimal growth at 37^{o}C with pH 7, agitation of 150 rpm and time period (5days). The results revealed the possibility to use these strain for the reduction of complex hydrocarbon in ecosystems where they accumulate and cause pollution problems. The highest rate of hydrocarbon degradation occurred when the bacterial strain is a biosurfactants producer. The selective strain produces biosurfactants which increase the interfacial area for contact to give improved uptake of hydrophobic substrates. Bacterial strains capable of degrading complex hydrocarbons, present in the environment, have a potential to be used as an effective tool for removing ecotoxic compounds. Furthermore, results indicated that the bacterial strain *Staphylococcus* sp could be potentially used in biodegradation of diesel oil in waste water and had a promising application in bioremediation of hydrocarbon contaminated environments.

Keywords: bioreduction, bioremoval, biosurfactant, diesel oil, *Staphylococcus sp*.

INTRODUCTION

Petroleum-based products are the major source of energy for industry and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. Soil contamination with hydrocarbons causes extensive damage of local system since accumulation of pollutants in animals and plant tissue may cause death or mutations. Toxicity of petroleum hydrocarbons is one of the primary reasons for remediation work. Diesel fuel keeps the world economy moving, from consumer goods moved around the world to the generation of electric power and to increase the efficiency on farms; diesel fuel plays a vital role in strengthening the global economy and the standard of living. The major uses of diesel fuel are farming, rail transportation, Marine shipping, off-road uses (e.g., mining, construction, and logging), electric power

*Corresponding Author Email: usharaniks2003@yahoo.com

generation, and military transportation. Because diesel fuel is used to move goods from manufacturer to consumer, its sales are linked to the economy strength. Diesel oil spills as a global problem due to the toxic compounds in the oil. The technology commonly used for the soil remediation includes mechanical, burying, evaporation, dispersion, and washing. However, these technologies are expensive and can lead to incomplete decomposition of contaminants. As microorganisms show broad range of mechanisms, there are still few mechanisms which are not known, consequently bioremediation is still measured as a growing technology (Rahaman *et al*., 2002; Ranjana *et al*., 2013 and Priya *et al*., 2014).

Biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which, petroleum and other hydrocarbon pollutants can be removed from the environment and is cheaper than other remediation technologies. One important requirement is the presence of microorganisms with the appropriate metabolic capabilities. If these microorganisms are present, then optimal rates of growth and hydrocarbon biodegradation can be sustained by ensuring that adequate concentrations of nutrients and oxygen are present and that the pH is between 6 to 9. The physical and chemical characteristics of the oil and oil surface area are also important determinants of bioremediation success. The microbial utilization of hydrocarbons depends on the chemical nature of the compounds within the petroleum mixture and on environmental determinants including n, iso, and cycloparaffins), and 25% aromatic hydrocarbons (including naphthalene and alkylbenzenes). The cheapest method of hydrocarbon reduction is by using microorganisms. So this subject matter is selected to identify an efficient organism which can remove diesel oil and it can be suggested for the bioremediation process.

MATERIALS AND METHODS

Sample collection

This study includes four types of samples collected from different places in and around Central University of Kerala (River side Transit campus), Kerala, SouthIndia, to isolate the hydrocarbon degrading bacteria. Soil sample extending from the ground surface to a depth of 5-10 cm were collected from hydrocarbon polluted soil from the automobile workshop at Neleswaram and non-contaminated soil from protected forest area (Nakravanam kavu). Water sample such as hydrocarbon polluted water from the harbor near Thaikkadappuram and non-contaminated water from non-polluted site of Thejaswini River. Samples were then transported to laboratory under sterile conditions. Commercial diesel was collected from local petroleum pump (Padannakad).

Isolation of Hydrocarbon degrading Bacteria

The bacteria were isolated from collected samples by pour plating the sample on Nutrient Agar Media (NAM) along with and without hydrocarbon source such as diesel. From the numerous colonies obtained on the NAM plates, colony having special characteristics is picked out and streaked on hydrocarbon containing nutrient agar medium. Pure isolated colony were taken from the nutrient agar plate and cultured on Mineral Salt Medium (MSM).Culture the bacteria on mineral salt media by pour plating method, Supplemented with 1% diesel as sole carbon source. (Mineral Salt Agar medium: K_2HPO_4- 0.7 g; $(NH_4)_2SO_4$ - 0.1 g; KH_2PO_4 - 0.3 g; $MgSO_4.7H_2O$; - 0.3 g; Agar -Agar - 2.2 g; Distilled water - 100 ml, pH - 7±0.2). The medium without hydrocarbons was sterilized by autoclaving at 121°C for 15 min. The medium was supplemented with 1% (v/v) diesel to serve as the only source of carbon and energy. The medium was incubated at 37°C for two

days. After the incubation period, the bacterial colonies that were grown on the medium was counted the colony and its characteristics was observed.

Assessment of bacterial growth using modified mineral salt agar media

Mineral salt media were modified for better growth of microorganisms by adding additional components with mineral components of the medium. Culture the bacteria on mineral salt media with each additional component by pour plating method. Diesel oil (DO) supplemented in different concentration like 2%, 4%, 6%, 8% and 10%. Better growths obtained on the DO media were considered for culture the hydrocarbon degrading bacteria for further study. In this study, additional components of Peptone - 5 g/l; Beef extract -1.5 g/l; NaCl 1.5g/l; Glucose -10g/l were added in mineral salt medium along with 10% diesel oil.

Morphological and Biochemical Characterization

Gram staining revealed that the morphological characters of the isolated bacterial strains. Spore staining shows weather the organism is spore producing or not. Motility test can determine the organism which is motile or non-motile. Catalase test was performed to check the ability of the isolated strains to degrade Hydrogen Peroxide and Oxidase test for the detection of presence of cytochrome oxidase enzyme. IMViC test is performed for the identification of the organisms. Carbohydrate utilization tests revealed that the ability of the isolated strains in fermentation of sugars like glucose, lactose, sucrose, and mannitol. The identification was done on the basis of morphological and biochemical characteristics as per Bergeys Manual of Systemic Bacteriology (Holt *et al*., 1994).

Screening of Biosurfactant producing isolates

The isolated microbes were screened for the production of oil degrading biosurfactant by the following methods.

Extraction of Biosurfactants

After 24 hours of incubation, the bacterial cells were removed by centrifugation at 5000 rpm for 20 minutes. Then added 20 µl of culture supernatant with 20 µl of mixture solution [equal volume of chloroform and methanol (2:1)] in a 96 well microtitre plates. This mixture was left overnight for evaporation and the results were observed. The biosurfactants settled as white colored precipitate.

Drop Collapse method

Biosurfactant production was screened using the qualitative drop collapse test. 2µl of diesel was added to 96 well micro- titer plates. The plates were equilibrated for 1 hour at 37°C and 5µl of the respective culture supernatant (obtained from the different soil sample) was added to the surface of the oil in the well. The shape of the drop on the surface was observed for 1 minute. If the culture supernatant makes the drop collapse, it indicated positive result for bio surfactant presence and if the drop remained as it indicated negative result.

Oil Spread method

The petriplate base was filled with 50 ml of distilled water. On the water surface, 20µl of diesel and 10µl of culture was added respectively. The culture was introduced at different spots on the diesel which is coated on the water surface. The occurrence of a clear zone was an indicator of positive result.

Emulsification Index

The emulsifying capacity was evaluated by an emulsifying index. The E24 of the culture samples was determined by adding 2 ml of diesel and 2 ml of the inoculums in a test tube and it was shaken for 2 minutes. Then water and diesel were added and shaken for 2 minutes, to obtain maximum emulsification and allowed to stand for 24

hours. This was taken as control. The percentage of the E24 index is calculated by the following formula.

$$E_{24} = \frac{Height\ of\ the\ emulsified\ layer\ (cm)}{Total\ height\ of\ the\ column\ (cm)} \times 100 \quad (1)$$

Microbial degradation of Diesel oil detection

Determination of growth of bacteria in diesel containing medium using UV-VIS Spectrophotometer

Isolated colony was inoculated to modified mineral salt broth along with 10% diesel then incubated for one day. This pure culture considered as sample and it was taken for hydrocarbon degradation study. Each sample was inoculated to each modified mineral salt medium and placed it to the shaking incubator at 150 rpm in 37^{o}C. Growth of the bacterium was measured by taking the O.D reading at 600 nm from the initial time (zero) to 9 days at regular intervals against mineral salt medium with diesel oil as blank.

Gravimetric Analysis

The amount of oil in culture was estimated using the Gravimetric method. Diethyl ether and acetone were taken in 1:1 ratio and was mixed with culture. The mixture was allowed to vaporize at room temperature. The oil residue obtained was weighed and taken as the gravimetric value for further calculation.

$$Percentage\ of\ diesel\ oil\ degraded = \frac{Weight\ of\ diesel\ oil\ degraded}{Weight\ of\ diesel\ oil\ present\ originally} \times 100 \quad (2)$$

where, the weight of diesel oil degraded = (original weight of diesel oil – weight of residual diesel oil obtained after evaporating the extract).

FTIR Analysis

The organic functional groups present in the sample of culture supernatant were determined using FTIR analysis. The analysis was carried out using FTIR-3500 spectrophotometer. To measure the absorption spectra, solvent extracted samples were dropped on the potassium bromide (KBr) crystal at a resolution of 4 cm^{-1} and measurement wave length range from 600 to 4000 cm^{-1} (Saher *et al*., 2011).

RESULTS AND DISCUSSION

Isolation of diesel oil tolerant bacteria from the sample

Collected samples were isolated by pour plating technique, from this pour plate colony the ones having special characteristics were selected and used for further studies. The total heterotrophic bacterial populations (THB) were found to be maximum in the soil sample collected from polluted and non-polluted sites. Countless number of bacteria was observed in both polluted (automobile workshop at Nileshwar) and non-polluted (Nakravanam Kavu) sites. THB from hydrocarbon polluted water sample (Thaikadapuram Harbor) was 210 $\times 10^2$ CFU/ml and the least number of THB population were found in samples collected from non-polluted water site (Thejaswini river) was found to be 3×10^2 CFU/ml (Table 1). Among the soil samples, the highest number of Hydrocarbon Utilizing Bacterial (HDB) population was found in the polluted soil sample collected from automobile workshop at Nileshwar (136×10^2 CFU/ml) and least number of HDB (50 $\times 10^2$ CFU/ml) found in soil sample from non-polluted site such as Nakravanam Kavu (Table 1). Similarly among the water samples, the maximum HDB counts (8×10^2 CFU/ml) were observed in polluted water sample (Thaikadapuram Harbor). In the non-polluted water sample (Thejaswini River) the maxium HDB counts was found to be 5×10^2 CFU/ml (Table 1).

Table 1. Total heterotrophic bacterial (THB) population and total hydrocarbon degrading bacterial (HDB) population from different locations

Locations	THB $\times 10^2$ CFU/ml (Nutrient Agar Medium)	HDB $\times 10^2$ CFU/ml (Nutrient Agar Medium)
Thejaswini River (NPW)	3	5
Thaikadapuram Harbor (PW)	210	8
Nakravanam Kavu (NPS)	Countless	50
Automobile workshop, Nileswar (PS)	Countless	136

*THB- Total heterotrophic bacteria; *HDB-Hydrocarbon Degrading Bacteria;
*Non polluted water (NPW), Polluted water (PW), Non Polluted soil (NPS), Polluted soil (PS)

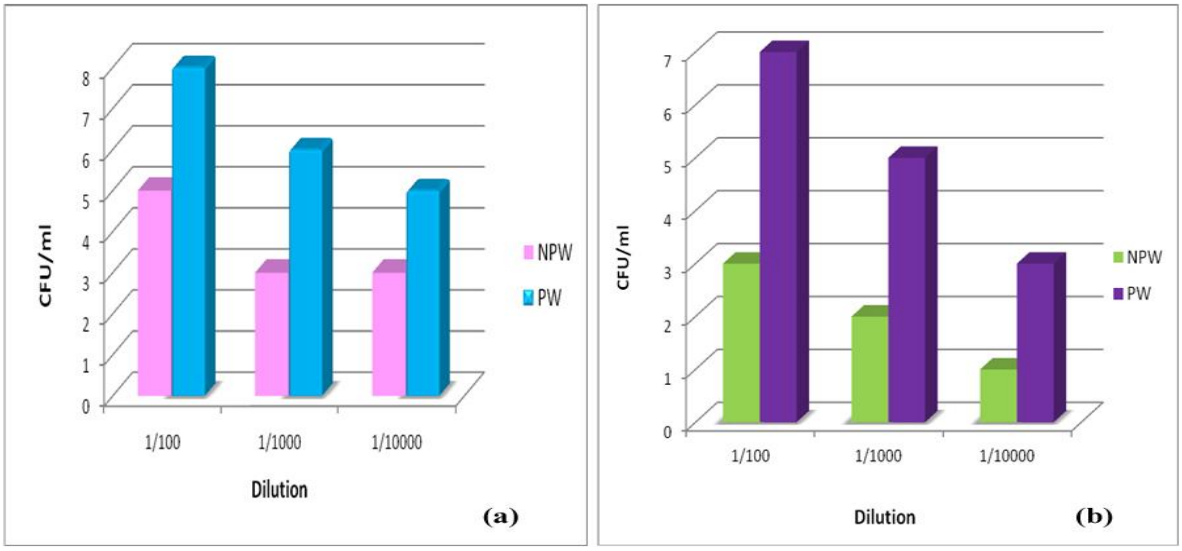

Fig. 1. Hydrocarbon utilizing bacterial population from water samples in nutrient agar medium with diesel and (b) in mineral salts agar medium with diesel

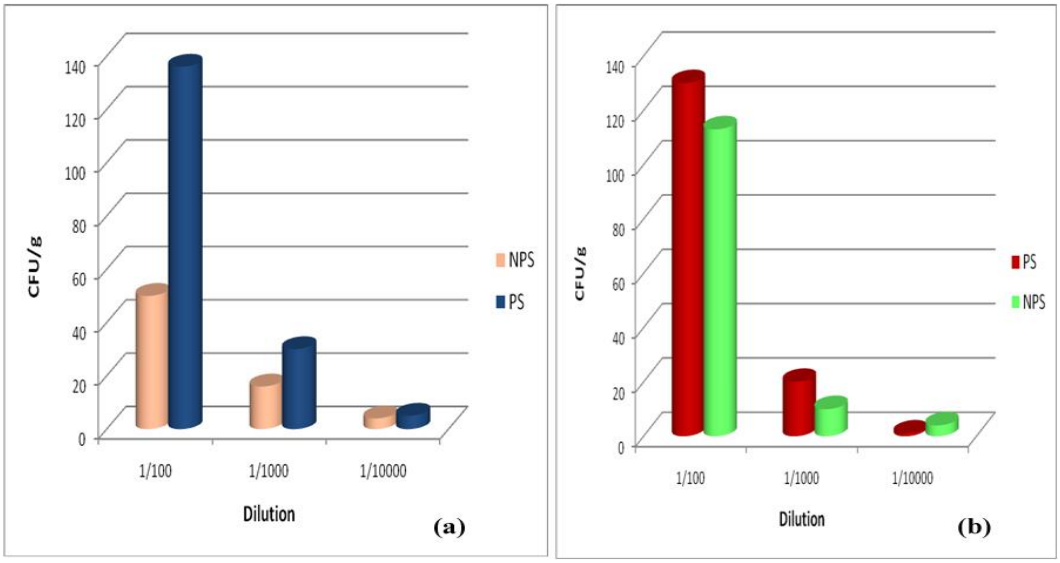

Fig. 2. Hydrocarbon degrading bacterial population from soil samples (a) in nutrient agar medium with diesel; (b) in mineral salts agar medium with diesel

From Figure 1, in comparison with the non-polluted water samples, the serial dilution shows higher bacterial population in samples collected from polluted water. The maximum number of hydrocarbon utilizing bacterial population was observed in 10^{-2} dilution in polluted water sample using nutrient agar medium with hydrocarbon (2%). From Figure 2, it can be interpreted that more hydrocarbon utilizing bacterial population were obtained from the soil sample collected from the polluted area than the non-polluted area. The figure can be revealed that the higher number of bacterial population is found in the polluted water sample compared with the non-polluted water sample when they cultured in mineral salt medium abounding with 2% of diesel oil. It showed that highest number of bacterial population observed in 10^{-2} dilution of polluted soil sample compared with the non-polluted soil sample. Similar studies on crude oil was conducted by Mili *et al*. (2009); Sharma and Pant (2001), Rahman *et al*. (2002). The results are comparable showing high occurrence of *Streptomyces*, *Nocardia* and *Rhodococcus* which were measures of incidence and growth of dominant group of actinomycetes.

Selection of Diesel oil utilizing bacteria

A total of eight bacterial isolates were screened for their growth on diesel containing media from oil contaminated sites. Five strains showed the best growth on nutrient agar plates with diesel up to 10%. When these strains were tested for their growth in Mineral salt medium with diesel up to 10% as sole source of carbon and energy, different levels of growth were observed. Majority of the isolates showed growth up to 10% concentration of the diesel tested. The selected bacterial strain showed rich growth in 10% diesel. So this strain was selected as diesel degrading organism for further study.

Morphology and Biochemical Characterization

The selective bacterial isolate was analyzed taxonomically. The colony morphology of this was abundant, opaque, golden tinch growth and round shape. The organism was found to be Gram positive and round shaped bacterium (Fig. 3). Based on the biochemical characteristics, the selected efficient bacterial species were identified as *Staphylococcus* sp, *Bacillus* sp and *Corynebacterium* sp at generic level (Table 2).

Table 2. Biochemical characteristics of selective bacterial isolate

Biochemical Tests	Results		
	Staphylococcus	***Bacillus***	***Corynebacterium***
Colony morphology on diesel oil And nutrient agar	Abundant, opaque, golden tinch growth	Cream, big, flat irregular white colonies; Abundant, opaque, white waxy growth	Grayish, granular growth
Gram stain	+, coccus	+, rod	+, rod
Spore	-	+	-
Motility	-	+	-
Catalase	+	+	+
Oxidase	-	-	-
Indole	-	-	-
MR	+	+	-
VP	-	-	-
Citrate	-	+	-
Glucose	+, Acid production	+, Acid production	+, Acid production
Lactose	Acid production	+, Acid production	-
Sucrose	-	+, Gas production	+
Mannitol	Acid production	+	-
Identified Bacterial Genus	*Staphylococcus*	*Bacillus*	*Corynebacterium*

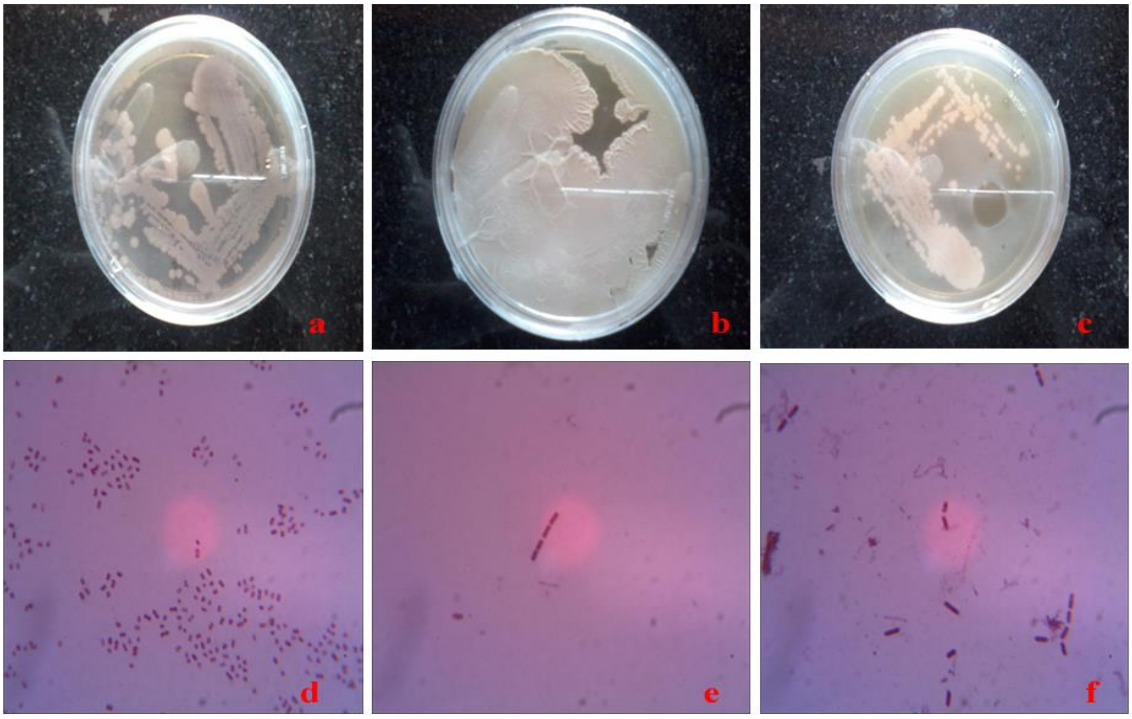

Fig. 3. Growth of selective bacterial strains (a) *Staphylococcus* sp , (b) *Bacillus sp*, (C) *Corynebacterium* sp on Nutrient agar with diesel and gram staining results at 100X

Screening of Biosurfactant producing isolates

Various screening methods are showed by the *Staphylococcus* strain; it has the ability to produce biosurfactants. Combination of various methods are required for effective screening of biosurfactants, a single method is not suitable to identify all types of biosurfactants.

Extraction of Biosurfactants

The experimental extraction of biosurfactants revealed that the *Staphylococcus* strain was observed to be positive (Table 3). The biosurfactants settled as white colored precipitate. Similar method was studied and reported by Vinoth Kumar *et al*. (2014).

Table 3. Evaluation of biosurfactant activity

S.NO	Method	Biosurfactant Activity
1	Drop collapse method	+ (Positive)
2	Oil spread method	+ (Positive)
3	Extraction of biosurfactants	+ (Positive)

Drop Collapse method

Surface activity of the compounds produced by *Staphylococcus* strain was detected by modified drop collapse method. However, the method is more qualitative than quantitative (Table 3). This isolate achieved collapse in drops in micro well plates. Drop–collapse technique is dependent on the principle at which a drop of a liquid involving a biosurfactant will collapse and spread over the surface of oil. Therefore, achieving collapse in drops was an indicator of biosurfactant production ability of the isolate. Similar methods were studied and reported by Youssef *et al*. (2004) and Chandran *et al*. (2010).

Oil Spread method

The study showed a high surfactant activity when observed in the isolated bacterial strain (*Staphylococcus sp*). Oil spreading assay was shown to be rapid and more sensitive for detection of surface active compounds. *Staphylococcus* strain demonstrated oil displacement activity towards diesel oil. As reported by

Rodrigues *et al*. (2006), higher diameters of cleared zone means more surface activity of the samples. Therefore, higher areas of oil displacement represented higher activities. It was observed that the oil displacement activity of the selected strain showed positive result (Table 3). The increase in biosurfactant activity was also analysed by other researchers (Ranjana *et al*., 2013; Vilma *et al*., 2011).

Emulsification Index

As Obayori *et al*. (2009) stated, release of biosurfactants is a strategy used by microorganisms to affect the uptake of hydrocarbon compounds. Therefore, measurement of emulsification activity (E_{24}) experiment was conducted for all of the bacteria which were successful at drop collapse and oil displacement tests. E_{24} was calculated using Eq. (1) as mentioned in the section of materials and method. The bacteria with emulsification indices higher than 50 % have been defined as potential biosurfactant producers. E_{24} (%) of selected, *Staphylococcus* sp strain examined for emulsification capacity, the experiment revealed that *Staphylococcus* sp strain was positive and showed 73% activity, followed by *Bacillus* sp (70%) and *Corynebacterium* sp (68%) as in Fig. 4. Similar studies were carried out by Rodriguez *et al*. (2012); Ranjana *et al*. (2013).

Detection of Diesel oil degradation

Bacterial growth estimation by using UV-VIS Spectrophotometr

Figure 5, shows that the growth of *Staphylococcus sp* in mineral salt broth supplemented with 10% diesel was studied by monitoring the optical density for a period of 9 days. An increase in growth rate (1.93-2.97 OD) of *Staphylococcus sp* was observed during the study period, maximum being 2.97 OD on 5th day of incubation, comparing the OD values of other isolates of *Bacillus* sp (1.92 OD) and *Corynebacterium* sp (1.43 OD). The culture reached stationary phase on 5th day of incubation. The culture reached decline phase on 7th day of incubation (Fig. 5). The growth of these isolates showed the utilization of hydrocarbons in the medium, it indirectly means that it is able to degrade the diesel oil in the medium.

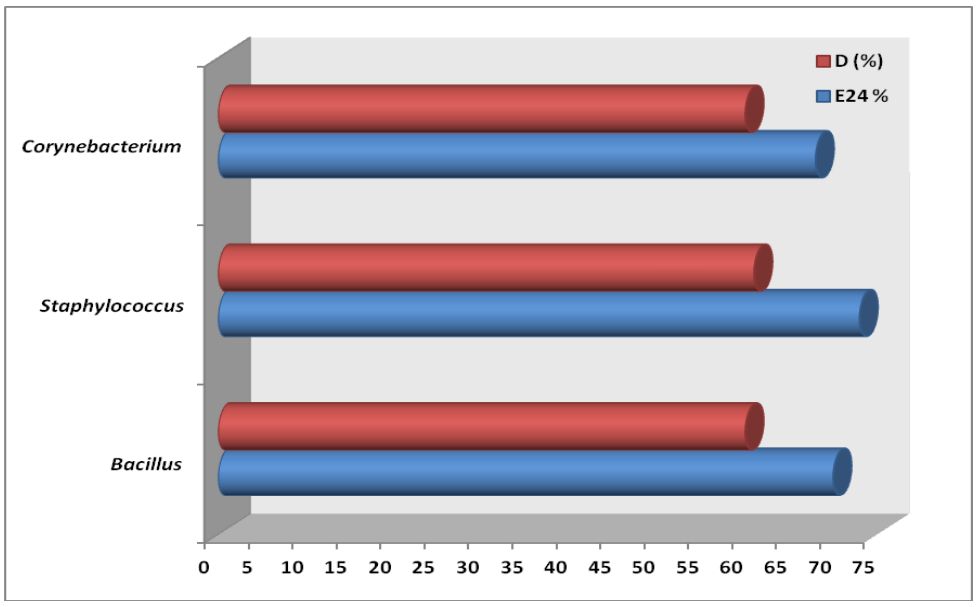

Fig. 4. Graphical expression of emulsification index (E24-%) and diesel utilization efficiency (D-%) using gravimetric analysis of *Staphylococcus sp*, *Bacillus* sp and *Corynebacterium* sp

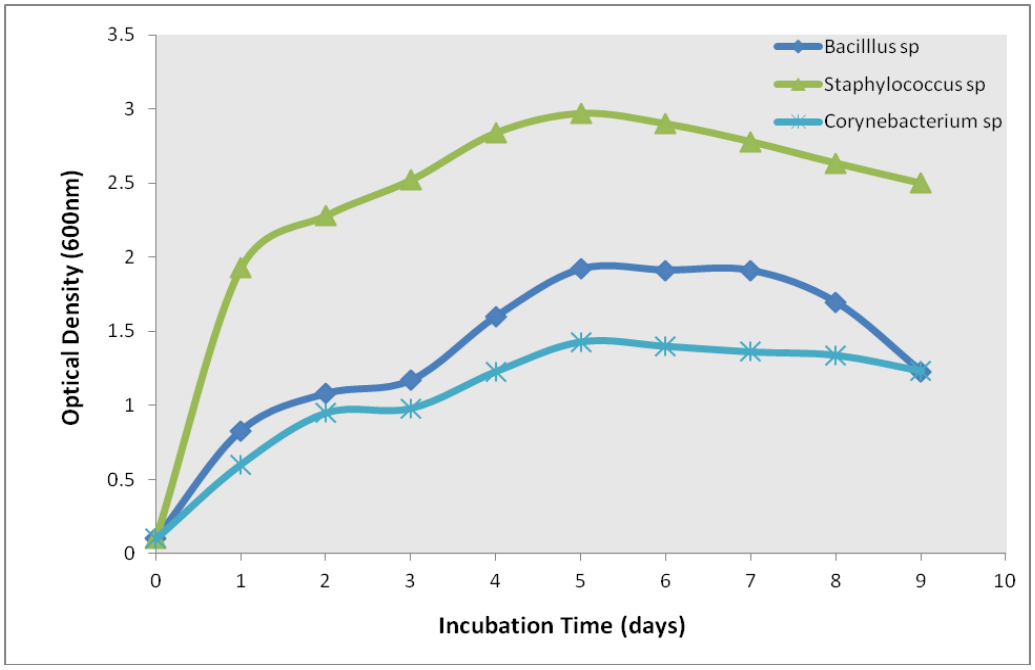

Fig. 5. Time course of growth for (a) *Staphylococcus*, (b) *Bacillus* and (C) *Corynebacterium* sp cultured in mineral salt broth supplemented with 10 % of diesel oil for 9 days

Gravimetric Analysis

Gravimetric analysis was used to determine the diesel degradation ability of the individual bacterial strains of *Staphylococcus* sp, *Bacillus* sp and *Corynebacterium* sp. Even though the gravimetric analysis is not as sensitive as GC analysis, it is comparatively helpful method for the preliminary determination. Therefore, the isolated bacteria found out to be successful in previous experiments where subjected to gravimetric analysis of diesel oil degradation. From Fig. 4, the degradation efficiency in terms of gravimetric analysis was observed to be higher in *Staphylococcus* sp (61%) followed by the *Bacillus* sps (60%) and *Corynebacterium* sp (60%). Residual diesel oil amounts in samples were calculated by using Equation (2). Olsen *et al*. (1999) reported 75% for the n-alkane fraction of total extractable petroleum hydrocarbons in diesel oil after 35 days in batch experiments. Lal and Khanas (1996) demonstrated degradation of the n-alkane fraction in the range of 43.18% over 15 days for crude oil from different sources in studies with pure cultures of *Acinetobacter calcoaceticus* and *Alcaligenes odorans.*

FTIR Analysis

The nature of biosurfactants as lipopeptide was further confirmed by FTIR analysis (Fig. 6). FTIR spectra showed peaks at 3238 cm^{-1} for N-H stretching, 2964 and 2856 cm^{-1} for $–CH_3$ asymmetric stretching, 2928 cm^{-1} for C-H asymmetric stretch of– CH_2- and $–CH_3$ in long alkyl chains, 2373 cm^{-1} for O-H stretching, 1749 and 1698 cm^{-1} for C=O stretching, 1662 cm^{-1} for peptide group in molecule, 1569 cm^{-1} for C=O stretching vibrations and N–H deformation in amines, 1268 cm^{-1} exhibits the C-N stretching in peptide bond (amide III band frequency). The presence of amide bond (617 cm^{-1}), N–H bending of primary amides (1658 cm^{-1}) confirms the peptide fraction of the biosurfactant. The nature of the biosurfactant produced by *Staphylococcus* sp was thus implied as a lipopeptide having a double bond in its fatty acid chain (Varadavenkatesan and Murty, 2013).

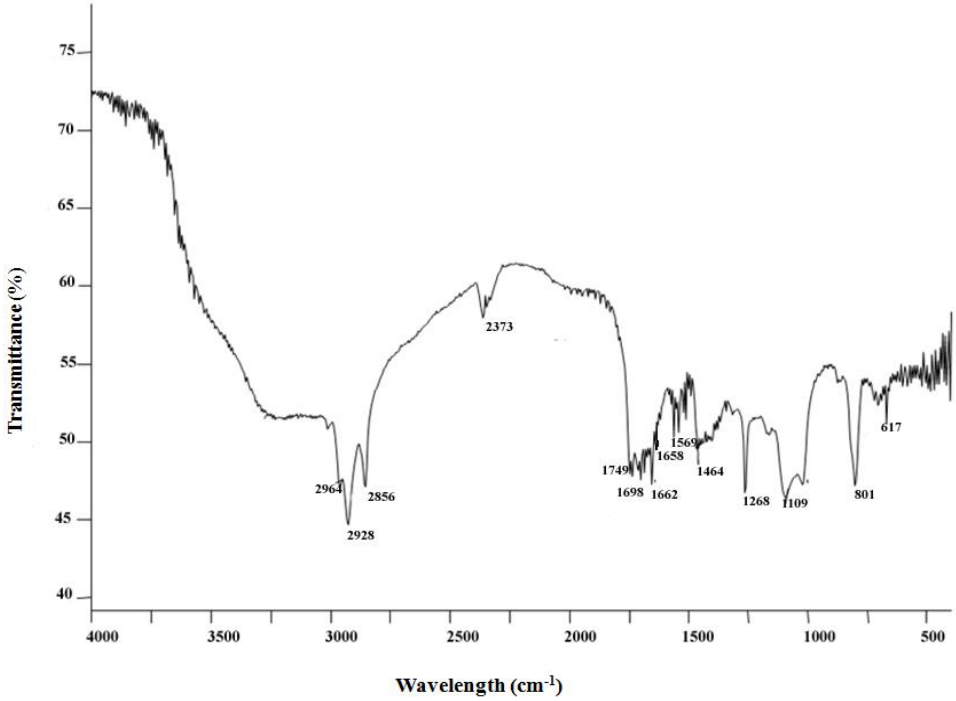

Fig.6. FTIR profile of diesel oil treated medium by *Staphylococcus* sp

From this study, the samples from hydrocarbon contaminated and non-contaminated areas were collected. Maximum hydrocarbon utilizing organisms were obtained in the sample from automobile workshop. From the collected samples, the isolates of the bacterial strain which shows good growth in the diesel containing medium was selected. Selected organisms are cultured on modified mineral salt media with diesel oil. The selected organism from polluted soil sample shows good growth within 24 hours compared to other isolates. The morphology and biochemical studies were carried out and it was found to be Gram positive cocci and it was identified as *Staphylococcus* sp according Bergys Manual of Determinative Bacteriology.

At an optimum condition of temperature (35^{o}C), pH (7) and agitation (150 rpm) after 5 days of treatment period, the optimum growth of *Staphylococcus* sp was found to be 2.97 OD at 600 nm using spectrophotometer. The gravimetric analysis for degradation of diesel oil revealed that, *Staphylococcus* sp showed 61% removal efficiency at higher concentration of diesel oil (10%). The extraction of biosurfactants experiment showed that the isolated bacterial strain was found to be positive. In addition, biosurfactants of the selected isolate was investigated for their diesel utilizing efficiency by drop collapse test and oil displacement activity, which was observed to be positive results. The isolated strain of *Staphylococcus sp* showed the emulsification index value of 73%. The FTIR analysis showed the changes in the organic functional groups of the diesel compound in the bacterial culture treated sample. To reduce the costs of the remediation, and enable more sites to be cleaned, these sustainable methods could be used more often in the future.

CONCLUSION

In conclusion, complex diesel oil is highly difficult to degrade. Among the bacterial isolates, *Staphylococcus* strain was observed to be maximum diesel oil utilizing ability (73% emulsification index) and change in the functional groups of the

compound (FTIR analysis). The strain showed optimal growth at 37^{o}C with pH 7, agitation of 150 rpm and time period (5 days). The results showed that the bacterial strain obtained was capable of using the complex diesel oil as a carbon and energy source in different environmental conditions. The strain *Staphylococcus* played a key role in the diesel oil utilization. In general, microorganisms produce biosurfactants to increase their interfacial area for contact to give improved uptake of hydrophobic substrates. *Staphylococcus* sp produce biosurfactants and this improved the breakdown of complex diesel oil. The highest rate of hydrocarbon degradation occurred when the bacterial strain is a biosurfactants producer. The results revealed that the possibility to use these strain for the reduction of complex hydrocarbon in ecosystems where they accumulate and cause pollution problems. The study observed that the potential bacterial strains isolated from hydrocarbon contaminated soil for bioremediation of hydrocarbon polluted area, spills as it suggests effective degradation of various fractions of hydrocarbons at broad range of concentration with respect to time period. Bacterial strains capable of degrading complex hydrocarbons present in the environment have a credible to be used as an effective tool for removing ecotoxic compounds. Furthermore, results indicated that the bacterial strain *Staphylococcus* sp could be potentially used in biodegradation of diesel oil in waste water and had a promising application in bioremediation of hydrocarbon contaminated environments.

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conflict of interest

The authors declare that there are no conflicts of interests regarding the publication of this manuscript.

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