Isolation of oil degrading bacteria from North India

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Abstract

Petroleum is very important energy resource. Petrol is used as a best suitable raw material in the chemical industry. It is generally used as a fuel and has led to the intensive economic development. Petroleum is also used as solvents in various industries like textile, pharmaceutical and plastic. It is made up of combining hydrocarbons and other organic compounds to form a complex mixture. The complex structure of hydrocarbon is responsible for its oxidizing property means more is the complexity in the structure of a hydrocarbon, the slowly it get oxidized. The bacterial samples were extracted from the oil contaminated soil to degrade the petroleum spillage, hence to reduce the pollution. The bacteria isolated belong to Staphylococcus spp. and pH range on which they were absorbed to be comfortable at 6- 8. The bacteria isolated have shown best growth in the range 30˚C to 40˚C. The bio surfactant and bio-emulsification activity was positive when compare to the control i.e *E. coli*. From the bio-surfactant and bio-emulsification activity it is clear that these bacteria can use petroleum oil as a substitute carbon source.

Introduction

Petroleum is known for possessing various constituents namely saturates, asphaltenes, aromatics and resins. Saturates hydrocarbons do not contain any double bonds, so they can be categorized on the basis of the chemical structures, e.g alkanes and cycloalkanes. Their percentage is highest among the constituents of crude oil. The next is aromatic hydrocarbons; they contain one or more aromatic rings. When we compare the saturated and aromatic fractions in petroleum, the polar compounds are present in resin and asphaltenes. Resins and asphaltenes are also known for their complex and unknown carbon structure. The structure contains nitrogen, sulfur and oxygen atoms in it. The compositional and physical properties of petroleum will depend upon the area from where it originated. The molecular weight of petroleum is also varies between very low to very high. The hydrocarbons attached also affects the biodegradation in two types. (1)molecules carry the groups or substituent and it cannot react with the accessible adaptive enzymes. (2)structure control thw compound in a physical state where microbes are not easily degraded. The degree of functional groups or substitution also affects the biodegradation of petrol. compounds groups like methoxy, sulfonate and amine ,along with the halogens linkages and carbon branched chains are relatively diligent. By the addition of some side chain of aliphatic group can be helpful in increasing adding some aliphatic side-chains can be helpful in increasing the vulnerability of cyclic hydrocarbons to microbial attack.

The industry belonging to the oil refineries and petroleum distillates plants are proved to be beneficial to association or society, but the pollution problem is also associated as the large amount of hazardous waste are produced. Mainly, during the exploration of oil, spills refining and transportation are reported the dangerous environmental pollutions. Today the major problem of environment is contamination of hydrocarbon emerge as activity done by petrochemical industry. Components of hydrocarbon belonging to the carcinogens and neurotoxic family members which are organic pollutants.

Exxon Valdez (1989), the Nahodka oil spill, the Erica spill (1999) and the Prestige spill (2002), these are result from the grounding of widely changes in terrestrial as well as marine ecosystem and they increase the attention of the chemists, environmentalists engineers and biotechnologists. Luckily , these oil degradation in the environment is possible through different types of method : biological, physical & chemical . These methods are mostly used for the soil remediation. It includes the evaporation, dispersion ,burying, mechanical, and washing. Decomposition of contaminations are incomplete due to the expensive methods.

In oil contaminated soils and general soil the pseudomonas species bacteria are observed abundantly. This species is able to adopt the different types of hydrocarbons. the aromatic compounds of gasoline and hydrocarbons strain are responsible for the degradation.NAH7 plasmid from pseudomonas putida are encoded the pathway for degradation of aromatic compounds of hydrocarbon (polycyclic).

Claude E. ZoBell (1946) analysed the activity of microorganism present in hydrocarbon. Katarina Malatova in 2005, published study about Screening microorganism present in hydrocarbon and helping in degradation. she was used the enrichment technique to isolate the 20 distinct species of microorganism from the different habitats in western new York state. cultivation of all the strain done in liquid media ,where the source of energy was crude oil as a single carbon. Only some of the Bacterial strains had capacity to degraded hydrocarbons. e.g. *Acinetobacter baumannii and Serratia marcescens, Pseudomonas sp*. For the microbial degradation of oil in biometric flask the carbon dioxide was used as a indicator in evolution experiment. According to study accomplished by Zhang *et.al.,* in 2010 he was concluded that from the oil field of Daqing, China .He collected the 38 bacteria through the enrichment cultivation. From 38 strains only 22 strains was consume by the source of single carbon energy and diesel oil. Other remaining 11 strains degrade by more then 70% of total petroleum hydrocarbons (TPHs) of diesel oil. 19 strains of the bacteria resemble from *Bacillus* species.The 87.5% seven strain of consortium is degraded by petroleum hydrocarbons (TPHs) crude oil

A. Swadgo *et.al,* concluded in 2014 about his study on isolation of hydro carbon degraders from the sample of oil waste water from this city Ouagadougou city (Burkina Faso). Darsa *et al*., in 2014 biodegradation of petrol was isolated by *pseudomonas aeruginosa* from the contaminated soil sample.The petrol with concentration 2.5%,5%,7.5%and 10% was used to grow the isolated strain of bacteria developing along with minimal broth which possessed the ability of degrading the petrol and using that petrol as a substrate for their growth. From 21isloated strains 7strains of bacteria were selected on the basis of more efficient growth rate in regard with crude oil and also it's capability of degrading hydrocarbon s. 16s rRNA gene sequencing showed that isolated strains were affiliated to *Bacillus, Pseudomonas* and *Halomonas* genera. After 10 days of cultivation in ONR 7 a mineral medium supplemented with crude oil degraders these strains showed a degradation rate of 80%, 60% and 59% respectively. These strain showed the production of bio surfactant and activity of high emulsification.

MATERIALS AND METHODS

The hydrocarbon degrading and contaminants indication as microbe were identified and collected from panipat refinery for subsequent identification and characterization of prevailing oil decomposing bacteria.. It is an oil refinery located in Baholi village, Panipat, Haryana, India and is known to be a seventh refinery belonging to Indian Oil Corporation Limited. The soil sample was collected from petroleum refinery in pre-sterilized polybags. Petroleum contaminated soil (200g.) was taken from a depth of 6 cm in order to avoid surface contamination. Collected sample was transferred to the laboratory and stored at 4˚C until processed for analysis.

1. **Isolation of bacteria and purification of the isolates:** Two test tubes were incubated with 5g of soil sample in distilled water. In test tube labelled as A added 20 ml of distilled water and in test tube labelled as B add 2ml petrol along with 18 ml distilled water. Both test tubes were kept for incubation at 37˚C for 2 days. After incubation of 2 days, take two new test tubes and 2ml culture from previously incubated test tubes in each and labelled as C and D. In C add 18 ml distilled water and in D add 16 ml distilled water and 2 ml petrol. These test tubes were again kept in incubator at 37˚C for 2 days. 3 Agar plates were prepared. One was control i. e only solified agar second was having nutrient agar and suspension and third was having nutrient agar (3%), petrol (1%) and 100ul suspension. This 100ul suspension and petrol was transferred to the agar plate using spread plate technique. On the agar plate surface 100 µl of petroleum was spreaded. As on this third agar plate having petrol the bacteria those uses petrol as their sole source of carbon will survive. Hence pure colonies of required isolate will be observed on this plate. The plates were incubated for 2-3 weeks at 37°c . After the period of incubation, only third plate showing the petroleum degrading bacteria on its surface. Now a pre-sterilized petriplates was taken and agar was poured and let it solidify in the laminar itself. 100ul petrol was suspended on it and let it dry. Streaking was done by the pure petrol degrading isolates from previous plate which was also being spreaded by petrol and suspension and kept for incubation for growth of petrol degrading bacteria. This newly prepared petri plate was kept for 24 hours at 37°c for incubation . Culture will be taken from this petri plate for further analysis.

3. Biochemical characteristics: Following biochemical tests were performed to check the identity of bacterial isolates

A) **Gram staining-** The Gram staining is always the first step for the bacterial identification.

B) **pH-** Nutrient agar was being prepared in 5 distinct 100ml flasks having (50ml agar in each).The pH of agar in each flask is adjusted to 4,5,6,7,8 and labelled as A,B,C,D,E respectively. Then these are autoclaved for 45 min. After autoclaving, the agar is poured in 5 seperate petri plates in sterile conditions in laminar and labelled and let it solidified in laminar itself. Bacterial culture is streaked on Petri plates having different pH ranges with sterlized inoculating loop. Now these Petri plates were kept for 24 hours at 37˚C for incubation to see the growth of bacteria.

C) Temperature- Nutrient agar was being prepared in a flask. It was poured in 5 different petri plates and solidified and these are labelled as 1,2,3,4,5. Streaking of bacterial culture was done on each petri plate. And the petri plates were kept for incubation at different temperatures -20˚,30˚,40˚,50˚,60˚ respectively for 24 hrs.

4. Biosurfactant activities - Three sterilized petri plates were taken and labelled as A,B,C. add distilled water was poured in each petri plate. Oil was added on top of water. A was control. In B, sample was added and in C, *E. coli* was added. petri plates were left undisturbed for 2 min. to check biosurfactant activity.

5. Bioemulsification activity - First of all the sample was centrifuged for 5 min. at 8000 rpm.Three sterilized test tubes were taken and labelled as A,B,C. and 5 ml of petrol was added. To test tube A, 5 ml distilled water was added. To test tube B, 5 ml culture was added and to test tube C, 5 ml supernatant was added and all three test tubes were vortexed for 2 minute to check emulsification activity.

RESULT

1. **Gram staining**- It was performed to identify bacteria based on stain it take. On staining gram positive stain of purple coloured cocci were observed. It may be *staphylococcus* as it forms clusters.
2. **pH**- The pH range on which the sample culture showed growth comes to be 6 to 8.i.e the bacterial culture shows growth only on petri plates having pH 6,7 and 8 respectively. The bacterial culture does not show any growth on the petri plates having pH 4 and 5. Also as the recent studies show the oil degrading bacteria have a pH range of 6 to 8 so isolates may be oil degrading bacteria.
3. **Temperature**- After incubation of 24 hours, the optimum temperature on which bacteria shows growth was 30˚ to 40˚.There was no growth on plates having temperature 20˚,

50˚and 60˚. Under this temp. the growth of strain is low. Temperature effect the biodegradation of hydrocarbons and showing their effect on chemical and physical composition of the oil .at a low temperature the volitization of toxic short chain alkanes is reduced and viscosity of oil and solubility of water increases. When temperature rise above the range of 30°c -40°c the rate of hydrocarbons metabolism is maximum ,in the same range of the temperature the hydrocarbons member of toxicity for microorganism is increased and metabolic activity is decreased. These findings supported our results.

4. **Biosurfactant activity** - Result for bio surfactant activity was positive as oil layer gets degraded in the Petri plate having sample culture. While in other two Petri plates oil layer does not gets degraded it remains suspended on the top of water. Oil degrading bacteria shows bio surfactant activity i.e wide range of organic compound sources(carbon & energy) are used for the growth of microorganism. In the insoluble form of carbon source like a hydrocarbon ,microorganism are diffused the cell by producing a different type of substances.(bio surfactants).so this shows that the isolates from the sample culture may be of oil degrading bacteria
5. **Bioemulsification activity** - The result for emulsification activity of bacteria also comes out to be positive. Oil layer in the tube having culture gets emulsified in the form of oil droplets

Discussion

On the basis of these results, it can be said that the sample may contain some strains of oil degrading bacteria. So the sample has been sent for 16S rRNA sequencing. The gene sequencing of 16S rRNA has been established as “gold standard” for taxonomic classification and bacterial identification .For bacterial identification the variable regions of 16S rRNA gene sequence are useful ,because the gene sequence provide a specific signature sequence of a species. The gene sequence methods are used to illustrate the new species that have never been cultured in laboratories scale. Due to the cost and technical issues the sequencing of 16SrRNA are used in less amount. For future work it need to be translate the information of gene sequencing from 16SrRNA into a suitable processing of biochemical testing. Thereby improving the efficiency and accuracy for identification of genotypic phenomenon for the betterment in smaller and larger clinical microbiology laboratories.

Figures

Fig. 1- sample collection

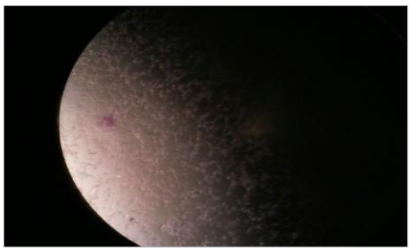

Fig. 2(a) - It is the picture of purple stained bacteria in petriplate. It is gram positive bacteria as it is retains purple colour.

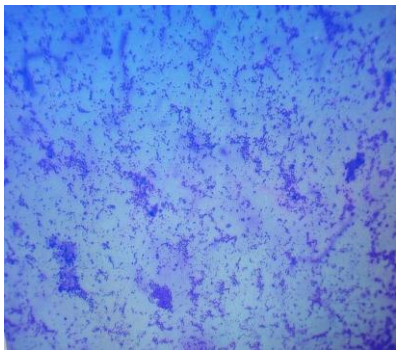

Fig.2(b)-Microscopic view of Gram staining result.

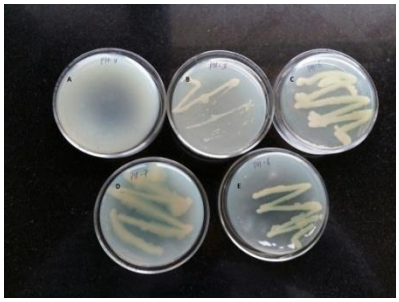

Fig.3- pH

A- pH 4: having no growth of bacteria

B- pH 5: having very less growth of bacteria

C- pH 6 : having accurate growth of bacteria

D- pH 7 : having maximum growth of bacteria

E- pH 8: also having growth of bacteria

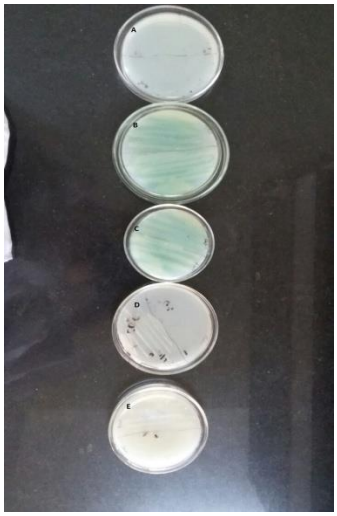

Fig.4-Temperature

Fig.5- Biosurfactant activities

A- Control

B- *E. coli* culture

C- Sample culture(strains of oil degrading bacteria)

Fig.6- Emulsification activity

A) Supernatant

B) Cells

C) Control

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