CHARACTERIZATION OF CULTURABLE BACTERIAL COMMUNITIES IN PETROLEUM HYDROCARBON CONTAMINATED SLUDGE OF OIL REFINERIES AND OIL EXPLORATION SITES

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Received October 15, 2013 *Accepted February 16, 2014*

ABSTRACT

Oil industries generate large amounts of oily sludge consisting of hazardous alkanes, aromatic hydrocarbons, NSO (Nitrogen, Sulphur and Oxygen) compounds and asphaltenes. Compared to conventional treatment technologies, microorganisms based bioremediation has been considered as an effective and economic strategy for the treatment of hydrocarbon contaminated wastes/sites. In the present study, eight different hydrocarbon contaminated sludge/soil samples, collected from oil -refineries/-exploration sites were characterized through chemical and microbial analyses. According to UPGMA analysis, the closest three samples (GR1, DB2 and DJ3) were chosen for further investigation. To determine culturable bacterial community composition in these samples 104 aerobic, heterotrophic bacterial strains were isolated and identified by 16S rRNA gene sequence analysis. Bacterial community in GR1 sample was composed of genera *Bacillus* (75%), *Burkholderia* (22%) and *Paenibacillus* (3%), while DJ3 sludge exhibited presence of *Pseudomonas* (47%), *Bacillus* (41%), *Stenotrophomonas* (9%) and Enterobacteria (3%) and DB2 sample showed the genera *Pseudomonas* (67%), *Bacillus* (20%), *Pandoraea* (7%) and *Kocuria* (6%). Metabolic potentials of bacterial isolates were characterized in terms of their growth with different nutrients, biosurfactant production, heavy metal tolerance and ability to utilize different petroleum hydrocarbons (Benzene, Toluene, Ethylbenzene, Xylene and crude oil) as sole source of carbon and energy. Overall observation indicated the bioremediation potential of isolated microorganisms indigenous to oily sludge.

Key Words : Bioremediation, Oily sludge, Microbial diversity, 16S rRNA gene, Biosurfactant

INTRODUCTION

Oil refineries and associated industries generate enormous amounts of oily sludge, which is a hazardous waste composed of alkanes, aromatic hydrocarbons, NSO compounds and asphaltenes.[1-3] The safe disposal of such waste has become a serious bottleneck for the refineries. The problem is not restricted only to the sludge generated after refinement of crude oil, but also during normal operation, exploration, leakage and spillage of crude oil resulting in contamination of locations such as oil wells, sumps and pits, tank batteries, gathering lines and pump stations.[4-6] The constituent compounds of petroleum hydrocarbons persist in the ecosystems have been considered to be recalcitrant due to their hydrophobic nature (low water solubility) and low volatility thus posing a significant threat to the environment. Sludge constituents, particularly the polyaromatic hydrocarbons (PAHs) have been known as the pollutants of great concern because of their toxicity, mutagenicity and carcinogenicity.[7] Owing to the increased public awareness and strict legal constraints on the release of environmental

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pollutants, there is a high demand for effective and affordable technology for the treatment of petroleum hydrocarbon contaminated wastes. Compared to the conventional treatment technologies, microorganisms based bioremediation technology has been considered as an effective and economically alternative strategy for the treatment of hydrocarbon contaminated wastes/sites. Communities of microorganisms rather than single strains have been considered as most important in bioremediation as their metabolic diversity and sometimes metabolic redundancy may contribute to process robustness. Powerful molecular biology techniques have revealed valuable insights on the structure and function of microbial communities associated with diverse contaminated environments. Culture dependent studies have explored the diversity in community composition and metabolic profiles of cultivable organisms in these environments. Such information on indigenous microorganisms has been considered to be useful in defining biostimulation and/or bioaugmentation based bioremediation strategies. Bioremediation of organic pollutants has been shown to be depleted in the presence of higher concentration of metals because of their impact on both the physiology and ecology of organic compound degrading microorganisms.[8] Many microorganisms have been isolated from various petroleum industry wastes and contaminated sites and have been reported to possess the ability to degrade a range of petroleum hydrocarbons.[9] Various hydrocarbon degradative pathways have been known to be present in many bacterial genera including *Rhodococcus*[10], *Pseudomonas*[11], *Acinetobacter*[12], *Alcanivorax*[13], *Burkholderia*[14], *Bacillus*[15] and *Gordonia*.[16] The indigenous bacterial population of contaminated sites has been shown to possess *in situ* bioremediation potential.[17]

AIMS AND OBJECTIVES

In the present study, we have isolated and characterized a number of aerobic, heterotrophic bacterial strains (104) from petroleum contaminated sludge of oil refineries and exploration site. The composition and metabolic properties of culturable bacterial communities have shown the innate potential of these microorganisms for bioremediation.

MATERIAL AND METHODS

Sample collection and chemical analysis

Eight different types of oil contaminated sludge/soil samples were collected from petroleum refineries of Indian Oil Corporation Limited (IOCL) at Guwahati and Digboi, Assam, India and oil exploration sites of Oil India Limited (OIL) at Dhuliajan and Jorajan, Assam, India. Chemical analyses (Oil and grease, aliphatic, total ion, and moisture contents) were done by Mitra S.K. Pvt. Ltd., Kolkata, India, using standard analytical techniques.

Enumeration and isolation of cultivable bacteria

The samples were diluted up to 10^{-4} and were plated on R2A agar (Himedia, India) and Minimal Salt Medium (MSM, g/L: K_2HPO_4 0.8, KH_2PO_4 0.2, NaCl 1.0, NH_4Cl 1.07, Na_2SO_4 1.07, $MgCl_2$. $6H_2O$ 0.43, $CaCl_2$. $2H_2O$ 0.03; pH 7.2) with 0.5% yeast extract. Colony forming units were determined after 7 days of incubation at 30 ºC. For anaerobic bacterial counts Anaerobic Agar (Himedia, India) was used and CFU/mg of samples was determined after incubating 16 days in anaerobic condition (Anaerobic Jar, Himedia, India). The bacterial colonies were further purified on R2A agar by repeated subculturing. Pure culture isolates were preserved at -80 ºC in 15 % glycerol.

Bacterial genomic DNA isolation and 16S rRNA gene analysis for taxonomic affiliation

Genomic DNA of the bacterial isolates was extracted using Promega Wizard Genomic DNA Purification Kit (Promega, USA) following manufacturer's protocol. Polymerase chain reaction (PCR) amplification of 16S ribosomal RNA gene (rRNA gene) was carried out using universal and bacteria specific primers 27F (5-AGAGTTTGATCMTGGCTC-3) and 1492R (5-GGTTACCTTGTTAC GACTT-3). The 25µl reaction mixture consisted of 10X buffer (New England Biolabs, USA), 200µM deoxy ribonucleoside triphosphate, 0.6 µM each of the primers (Sigma, USA), 100ng of template DNA and 1.0 U *Taq* polymerase (New England Biolabs, USA). Cycling conditions for the amplification reactions were: initial denaturation of 5 min at 94 ºC, 30 cycles of denaturation (1 min at 94 ºC), annealing (45 seconds at 58 ºC),

extension (1 min 30 seconds at 68 ºC) and final extension for 10 min at 68 ºC. The amplified product was examined by horizontal electrophoresis in 1% agarose (Sigma). Amplified 16S rRNA genes were sequenced and the sequences were analyzed using NCBI BLASTn and RDP (Ribosomal Database Project) classifier programs.

Metabolic characterization of the isolates

Growth potentials of bacterial isolates were tested in different liquid media like LB, R2A, MSM supplemented with either yeast extract (0.5%) or carbon cocktail [mixture of glycerol, glucose, sodium pyruvate and sodium gluconate (each 1.25%, w/v)]. Microbial strains were also tested for their ability to utilize different petroleum hydrocarbons [BTEX (Benzene, Toluene, Ethyl benzene and Xylene in equal molar ratio), 200 ppm and Crude oil, 1%] as sole source of carbon during growth in liquid MSM at 30 ºC for 7 days. Biosurfactant production was estimated by determining emulsification indices as described by Cerqueira et al.[18] Biosurfactant production was observed by growing the isolates in LB broth for 48 hours at 30 ºC followed by vortexing the cell free spent media with commercial diesel in a proportion of 1:1. Emulsification was observed after 24 hours and expressed as E24 value. Stable emulsions were considered as the positive result. Heavy metal (lead, cadmium and nickel) sensitivity of the isolates was investigated following growth (30 ºC, 7 days) on agar plates of MSM supplemented with 1mM of lead nitrate ($PbNO_3$) or cadmium nitrate ($CdNO_3$) or nickel chloride hexahydrate ($NiCl_2$. $6H_2O$).

RESULTS AND DISCUSSION

Sample characterization and enumeration of bacterial counts

Oil contaminated sludge and soil samples were analyzed in terms of their oil and grease, aliphatic hydrocarbon, total ions and moisture contents. A number of aerobic and anaerobic bacteria were isolated using R2A, MSM and Anaerobic Agar. The chemical characteristics and microbiological results were summarized in **Table 1**. Oil and grease content has been considered to be the most determining hazardous factor of the samples. It was found that the range of oil and grease content varied from 0.3 to 90.3% (w/w). GR3 sludge showed highest oil and grease content (90.3%), while DJ1 sample has the lowest value (0.3%) regarding this. Similarly, amount of aliphatic hydrocarbon was also highest in GR3 sample (1.21%), while J3 and DB4 samples showed lowest aliphatic content (0.01%). This could be attributed to the nature of the samples as GR3 was an oily sludge obtained from Guwahati refinery, while DJ1 sample was crude oil contaminated soil from Dhuliajan Oil field. Total ion content was comprised of various anions and cations including phosphate, sulphate, nitrate, nitrite, chloride, nickel, arsenics, lead, zinc, iron (III), iron (II), sodium, potassium, cadmium, chromium, cobalt and vanadium which was in the range of 0.23% to 1.5%. Moisture content was highest in DB3 sample (52.66%) followed by DB4 (38.62%), J3 (25.42%), DJ1 (22.84%), GR3 (9%), DB2 (6.6%), DJ3 (6.51%), and GR1 (4.52%) samples. Aerobic CFU counts/mg sample after 7 Days in R2A medium is 8.0 X 10^2 to 8.75 X 10^4 with GR1 having the lowest and J3 with highest bacterial counts. Similarly, when the same samples were plated on MSM with yeast extract, 2 X 10^2 to 5.25 X 10^4 CFU/mg was obtained, GR3 is having the lowest and J3 having the highest bacterial counts. On the other hand, anaerobic bacterial count was observed to be in the range between 2 X 10^2 and 3.2 X 10^4 CFU/mg again with GR3 showing the lowest followed by GR1 and J3 scoring the highest **(Fig 1)**. The observed variations in CFU counts among the samples could be due to the variation in the chemical nature of the sludge/soil samples.

All the samples were further compared using Unweighted Pair Group Method with Arithmetic Mean analysis (UPGMA) on the basis of their relatedness in terms of hydrocarbon content and their CFU counts generated by MVSP (Multi Variate Statistical Package) software. It was found that samples GR1, DB2 and DJ3 were closely related **(Fig. 2)** and hence these three samples were considered for further study. Simultaneously, the study is limited to 35 isolates from GR1 sample, 35 isolates from DB2 sample and 34 isolates from DJ3 sample.

Table 1 : Chemical and microbial parameters of the samples

Sample Id	Sites	Oil and grease content (% w/w)	Aliphatic content (% w/w)	Total ions content (% w/w)	Moisture content (% w/w)	No. of aerobic isolates	No. of anaerobic isolates
GR1	Guwahati Refinery, IOCL	28.6	0.55	1.5	4.52	35	7
GR3	Guwahati Refinery, IOCL	90.3	1.21	0.23	9.0	8	6
DJ1	Duliajan Oil fields, OIL	0.3	0.04	0.85	22.84	16	ND
J3	Jorajan Oil fields, OIL	0.4	0.01	0.63	25.42	22	ND
DB2	Digboi Refinery, IOCL	29	0.03	1.26	6.6	35	33
DB3	Digboi Refinery, IOCL	0.9	0.07	2.01	52.66	14	ND
DB4	Digboi Oil Fields	12.7	0.01	1.27	38.62	25	25
DJ3	Duliajan Oil fields, OIL	37.8	0.02	1.29	6.51	34	8

Culturable bacterial community composition

Culturable bacterial community of the selected samples were found to be represented by members of *Gammaproteobacteria*, *Firmicutes* and *Betaproteobacteria* along with a very small representation of *Actinobacteria*. Bacterial community of GR1 sludge was found to be dominated by Gram positive high GC bacteria *Bacillus* (75%) followed by Gram negative *Burkholderia* (22%) and Gram positive *Paenibacillus* (3%), whereas DJ3 sample was comprised of mostly *Pseudomonas* (47%), *Bacillus* (41%), *Stenotrophomonas* (9%) and a small fraction of *Enterobacteriaceae* members (4%).

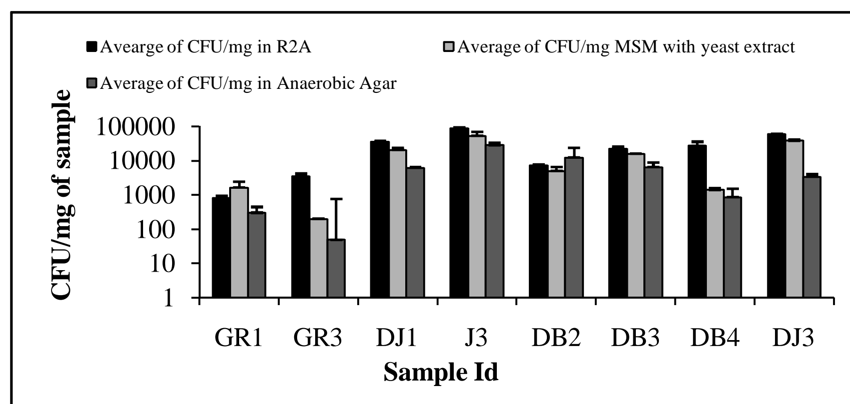


Fig. 1 : Colony forming unit counts in different media

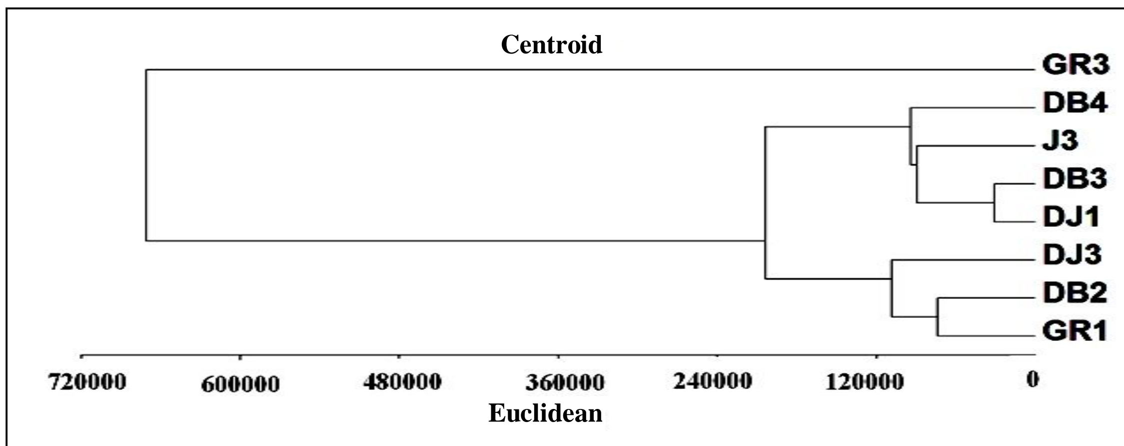
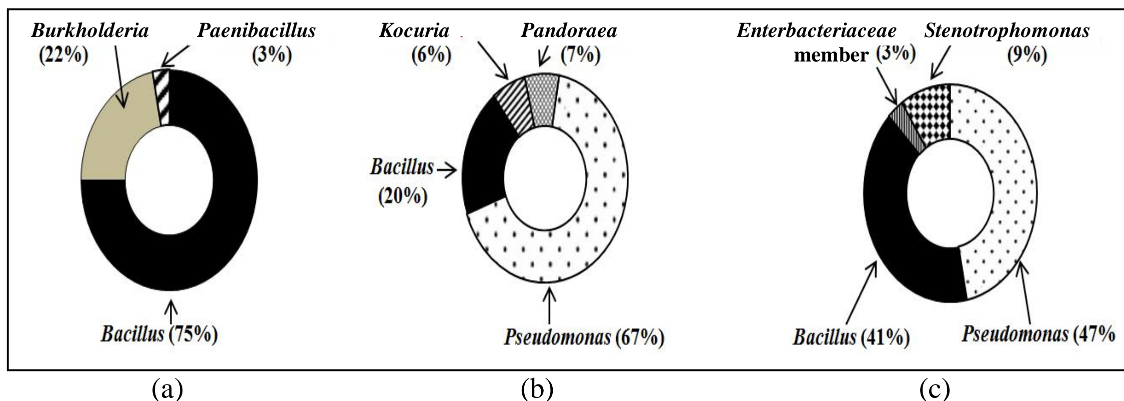


Fig. 2 : UPGMA analysis of the samples with their relative closeness on the basis of their hydrocarbon and CFU contents

Cultivable bacterial community in DB2 sludge was comprised of *Pseudomonas* (67%), *Bacillus* (20%), *Pandoraea* (7%) and *Kocuria* (6%) **(Fig. 3)**. It has already been reported that petroleum hydrocarbon contaminated sites were populated with *Pseudomonas, Bacillus* and *Burkholderia*, but reports on *Enterobacteriaceae* members were limited.[19] *Stenotrophomonas* isolated from hydrocarbon-contaminated environments have been reported for being able to degrade aliphatic and aromatic hydrocarbons.[18] Less culturable diversity as observed in GR1 sample could be attributed to the huge contamination of the site with recalcitrant hydrocarbons.[20]



(a) (b) (c)

Fig. 3 : Culturable bacterial community composition in GR1 (a), DB2 (b) and DJ3 (c) samples

Metabolic characterization of the isolates

All the isolates have shown to have the capability to grow in an array of growth media including LB, R2A, MSM with 0.5% yeast extract and MSM with 5% carbon cocktail **(Fig. 4)**. Noticeably, all the isolates from DJ3 showed different hydrocarbon (BTEX and crude oil) utilization potential, while in case of GR1 and DB2 samples, 25% to 35% of the isolated strains showed the ability to utilize crude oil as sole carbon source. Members of the genus *Pseudomonas* have been reported to utilize components of BTEX mixture as well as crude oil during growth.[21-23] Crude oil utilization by the isolates clearly indicated the bioremediation potential of indigenous bacterial population within the sludge samples. Biosurfactants have unique property of enhancing the bioavailability and accessibility of hydrophobic chemicals by forming stable emulsions and lowering the surface tension of the immiscible liquids. Thus, biosurfactant production has crucial role in degradation of oily sludge constituents.[24] Interestingly, all the isolates of DB2 showed the ability to produce biosurfactant, while surfactant production was observed in 35% and 37% of DJ3 and GR1 isolates, respectively **(Fig 4)**.

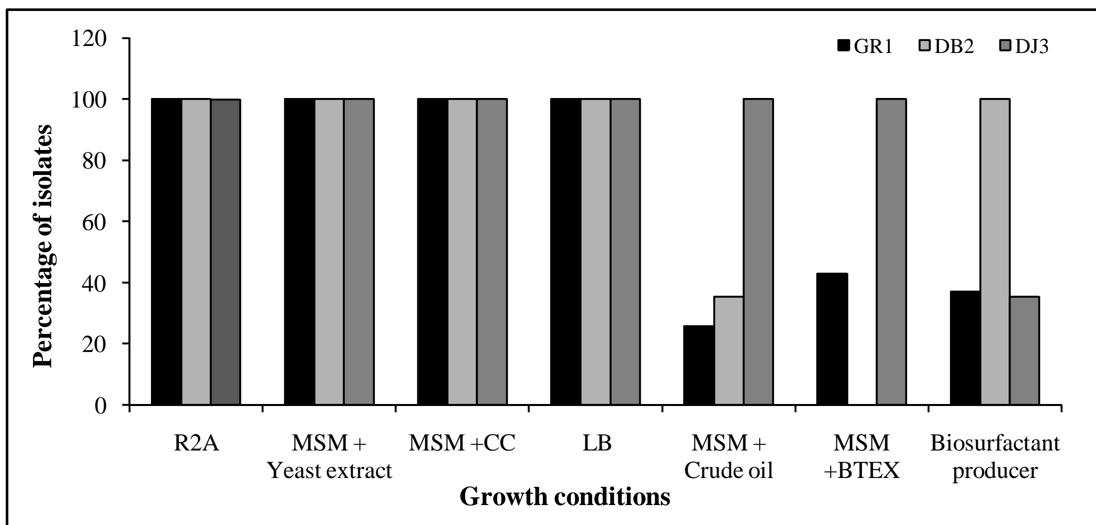


Fig. 4 : Growth under different nutritional conditions and biosurfactant production

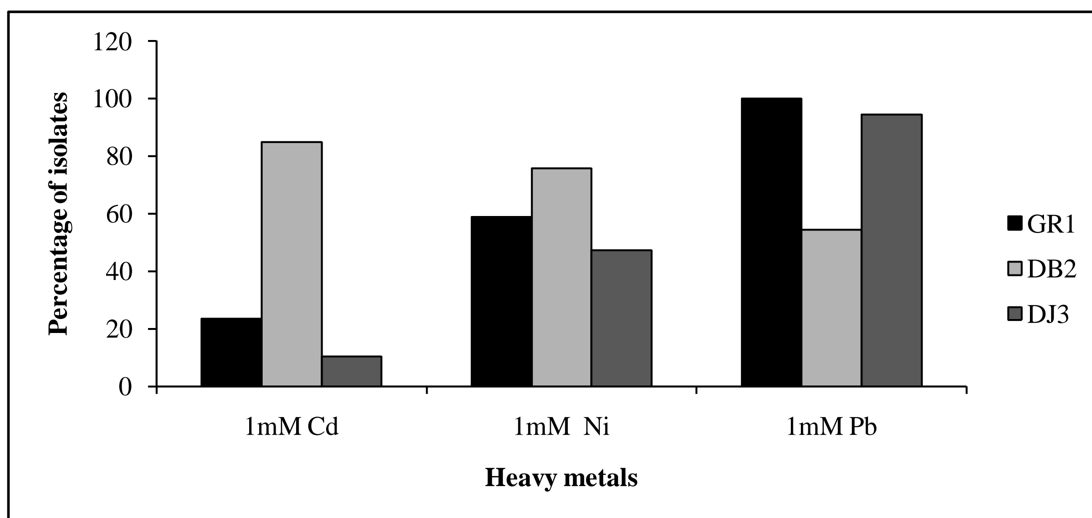


Fig. 5 : Different heavy metal tolerance of the bacterial isolates

Heavy metal tolerance of the isolates

Microbial bioremediation potential have been found to be directly related to their heavy metal tolerance capacity as the toxic concentrations of heavy metals have been known to reduce the sustainability of the potential biodegrading organisms of petroleum hydrocarbons.[25] All the samples in our study have reasonable amount of different heavy metals and therefore, the isolates were tested for their heavy metal sensitivity. All the GR1 isolates showed tolerance to 1mM Pb during growth, while for DJ3 and DB2 samples, 95% and 55% isolates, respectively showed resistance to 1mM Pb. Almost 50 to 75% of the isolates showed tolerance for 1mM Ni. Interestingly, around 85% of DB2 isolates exhibited tolerance to 1mM Cd, while only 12% of DJ3 isolates and 39% of GR1 strains could resist Cd at 1mM concentration **(Fig. 5)**.

CONCLUSION

The current study is focused on isolation and characterization of indigenous bacteria from oil refinery sludge and contaminated soil from oil exploration sites of Assam to assess the microbial metabolic potential towards bioremediation of contaminants present in those samples. Taxonomical identity was determined by 16S rRNA gene sequencing and the results showed the presence of *Bacillus*, *Pseudomonas*, *Burkholderia*, *Stenotropho - monas, Paenibacillus, Pandoraea*, *Kocuria*

and *Enterobacteria* in those samples. Several isolates showed their ability to utilize different petroleum hydrocarbons including BTEX and crude oil as sole source of carbon and energy as well as to withstand exposure to heavy metal stress during growth. Overall observation reflected the innate potential of indigenous microorganisms for bioremediation of oily sludge.

ACKNOWLEDGEMENT

This work was funded by Department of Biotechnology, Govt. of India under the NER Twinning programme (Project No. BT/226/NE/TBP/2011). We are thankful to NIT Durgapur and IIT, Kharagpur, respectively for fellowship. Authors are thankful to authorities of Guwahati and Digboi refineries, Indian Oil Corporation Limited, Assam and Dhulijan oil fields, Oil India Limited, Assam for extending their support for sampling.

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