Characterization of benzene, toluene, naphthalene and acenapthene degrading bacteria isolated from petroleum oil contaminated soil sediments located in Chhattisgarh and evaluation of their antibiotic and heavy metal resistance

ABSTRACT
of

THESIS

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
LUCKNOW

BABASAHEB
BHIMRAO
AMBEDKAR
UNIVERSITY

LUCKNOW

प्रज्ञा शील करुणा

ESTABLISHED 1996

FOR THE DEGREE OF

Doctor of Philosophy

IN

BIOTECHNOLOGY

Submitted by

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Enrolment Number: 441/11

2017

ABSTRACT

As economic aspects are getting more considered in remediation processes, the more inexpensive bioremediation methods e.g. in situ biostimulation and bioaugmentation techniques are preferred. However, to evaluate the applicability of these bioremediation techniques in a petroleum hydrocarbon contaminated environment, it has to be known whether the microbial community of the contaminated environment has the metabolic potential to eliminate the contamination.

Due to the fact that Chhattisgarh has a notable oil collection centres, renowned steel production industries, aluminum production plants and power plants there is lot of hydrocarbon and heavy pollution around these industries. Since remediation of these sites is considerably costly, development of a microbial soil inoculant for bioaugmentation purposes could be an appropriate approach to treat the contaminated sites in Chhattisgarh. Therefore, the major goal of this study was to obtain a strain collection of hydrocarbonoclastic bacteria able to exert an outstanding degradation potential against high levels of hydrocarbon pollutants even within heavy metal impacted environments. In addition, our aim was to investigate the heterotrophic and fuel degrader counts among ten geographically close petroleum hydrocarbon contaminated sites. Furthermore, we determined the heavy metal tolerance and antibiotic resistance of isolated and identified aerobic hydrocarbon-degrading bacterial strains.

OBJECTIVES

1. Site description and soil sampling

2. Physico Chemical Analysis (Organic and Inorganic) of soil samples

3. Enrichment, purification and culturing of hydrocarbon degrading bacteria

4. Estimation of total heterotrophic and fuel degraders by MPN method

5. FTIR analysis of soil samples

6. Characterization of selected isolates

7. Degradation potential of the characterized isolates by HPLC and UPLC

8. Antibiotic resistance pattern of characterized isolates

9. Heavy metal resistance pattern of characterized isolates

METHODOLOGIES

Site description and soil sampling:

It was done in 10 different sites located in Chhattisgrah which were both petroleum oil and heavy metal contaminated. Soil samples were collected from ten different areas in Chhattisgarh (Table 1). The selected areas were from Raipur (21.2514^0N, 81.6296^0E), Bhilai (21.1938^0N, 81.3509^0E), Bilaspur 22.076^0N, 82.139^0E, Korba (22.3595^0N, 82.7501^0E) and Raigarh (21.8974^0N, 83.3950^0E).

Physico Chemical Analysis (Organic and Inorganic) of soil samples:

Soil pH of samples was determined by following the SR ISO 10390-1999 standard (Muntean and Rusu, 2011). Moisture content of soils (expressed in %) was determined according to Damian et al. (2008); available potassium and organic carbon content was analyzed by Soil testing Kit (HiMedia) while the amount of phosphate and nitrate content was analyzed according to (Hooda and Kaur, 1999).

Enrichment, purification and culturing of hydrocarbon degrading bacteria:

Diesel-oil degrading bacteria were isolated using enrichment containing: 40 ml BBH mineral broth medium supplemented with 1 ml diesel and 4.0 g of contaminated soil sediment. After 2 weeks of incubation at 32^0C, the enriched cultures were serially diluted and inoculated onto BBH agar plates. The lid of Petri-dishes contained 250 ml of sterile diesel-oil as sole source of carbon and energy. Colonies with different morphologies were selected as candidate petroleum hydrocarbon degrading strains and were maintained on standard Nutrient Agar (HiMedia).

Estimation of total heterotrophic and fuel degraders by MPN method:

Each soil sample (100 g) was dissolved in 250 ml of deionised water and kept to stand for 30 mins after vigorous shaking in sterile conditions. The sediments were collected and air dried for all the future experiments. Aerobic heterotrophic and hydrocarbon degrading bacterial counts were estimated using the most probable number (MPN) method using 96 well microtitre plates using two different media. For aerobic heterotrophic count Bacto Bushnell Haas broth + Glucose was used and for hydrocarbon degrading count BBH broth + Tetrazolium Violet (2,5-diphenyl -3- α napthyl tetrazolium chloride) was used as media. Tetrazolium is reduced to a dark purple colored derivative formazan on microbial respiration.

FTIR analysis of soil samples:

The infrared spectra were recorded on Thermo Scientific USA. The spectra were scanned in the 400–4000 cm^{-1} range. The spectra were obtained using potassium bromide (KBr) pellet technique. Potassium bromide (AR grade) was dried under vacuum at 100 °C for 48 h and 100 mg of KBr with 1 mg of sample was taken to prepare KBr pellet. The spectra were plotted as intensity versus wavenumber.

FAAS analysis of soil samples:

All the soil samples were subjected for heavy metal concentration after the acid digestion of soil sample: (Followed from EPA3050b)

Characterization of selected isolates:

The selected bacterial isolates were Sequenced by Miniprep kit (Qiagen) and sequenced by using Big Dyeterminator with an automated capillary sequencer (Applied Biosystems) in NBRI, Lucknow. Sequences obtained were identified using NCBI BLAST program and the sequences submitted to NCBI under accession numbers KX371250-54.

Degradation potential of the characterized isolates by HPLC and UPLC:

This method was setup for four time intervals for each identified strain and hydrocarbon. The test

media consisted of 20 ml BBH supplemented with one of the filter sterilized (0.2 μm) hydrocarbons at a final concentration of 200 mg/l for naphthalene and acenapthene whereas 0.1 % for benzene and toluene set after optimization experiments. Incubation was done at 35^0C for 10, 20, 30 and 40 days at 150 rpm. Samples were collected at regular intervals of time and the residual hydrocarbons were extracted according to Vilas Patel et.al. (2012) and analyzed by HPLC (Benzene and Toluene) and UPLC (Napthalene and acenapthene).

Antibiotic resistance pattern of characterized isolates:

Sensitivity of selected strains against antibiotics was assayed by the Kirby Bauer's disc diffusion method using Muellere Hinton agar (Bauer et al., 1996; CLSI, 2013). Used antibiotic discs (HiMedia) belonged to the following antibiotic groups: cephalosporins (cefuroxime sodium-CXM30, cefoperazone-CFP75, cefotaxime-CTX30,); tetracyclines (tigecycline-TGC15); penicillins (penicillin G-10U, piperacillin-PRL100, amoxyclav-30); quinolones (norfloxacin-NOR10); carbapenems (imipenem-IPM10). Regarding to antibiotic resistance, strains were divided into three groups (resistant-R, intermediate resistant-I, susceptible-S) according to the diameter of the inhibition zone taking into account CLSI interpretive standards (CLSI, 2013). All the experiments were done in triplicates.

Heavy metal resistance pattern of characterized isolates:

Heavy metal resistance of strains was tested in nutrient broth tubes containing different concentrations (0.25, 0.5, 1.0, 2.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 mM) of $CdCl_2$, $CuSO_4.5H_2O$, $Pb(NO_3)_2$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, $C_8H_4K_2O_{12}Sb_2.3H_2O$, $SnCl_2.2H_2O$, $K_2Cr_2O_7$, $NiSO_4.6H_2O$, $HgCl_2$, $FeSO_4.7H_2O$ (HiMedia). Stock solutions were prepared in deionized water and were also filter sterilized before inoculation. All tests were done in duplicates and the results were evaluated visually for growth against heavy-metal free control cultures and blank tubes.

RESULTS

Physico chemical properties of soil samples:

The pH of the soil samples varied from 7.57 (HPRB) to 8.5 (KNPP, RJSP, BSP) which is alkaline. The soil temperature varied from 36.2 (KNPP) to 37.5 (CFK). Moisture content was found to be most for HPRB (7.5 %) and least for APR (0.2 %). Inorganic phosphate , nitrate and potassium contents were less when compared to uncontaminated soil standard values. The organic carbon content was high or medium for all the samples except CFK.

Total aerobic heterotrophic and hydrocarbon degrading bacteria counts of soil samples:

The total heterotrophic count of bacteria was highest in sample sites BROD, HPRR and BSPOCC and least in CFK. Hydrocarbon degrading bacterial count was less than the total heterotrophic count in most of the samples. In case of benzene degraders the count was maximum for BROD, HPRR, and APR and least for CFK whereas in case of toluene it was highest for BROD, HPRR and BSP and least in CFK. In case of naphthalene degraders the count was maximum for BROD, HPRR, and APR and least for IORR which followed the same trend in case of acenapthene degraders.

FTIR analysis of soil samples

From the analysis of FTIR peaks it is clear that all the soil samples contain aromatic compounds other than alkanes, alcohols, phenols, acids, esters and ethers. The above data was inferred through FTIR absorbance peaks.

Taxonomic Identification of the bacterial strains

The unknown bacterial cultures were identified as members of the phylum Firmicutes consisting predominantly of gram positive Bacillus and Aneurinibacillus species

Table **:** Taxonomic identification of the isolated bacterial strains.

Strain	**Gene bank accession number**	**Sequence alignment**		**Nearest phylogenetic neighbor (Gene bank accession number)**
		No. of nucleotides	Identity, %	
Aneurinibacillus aneuriniliticus strain RSP	KX371250	730	97	*Aneurinibacillus aneuriniliticus* strain NBRC 15521 (NR112639)
Aneurinibacillus migulanus strain KTPP	KX371251	1187	97	*Aneurinibacillus migulans* strain NBRC 15520 (NR113764)
Aneurinibacillus migulanus strain BROD	KX371252	865	98	*Aneurinibacillus migulans* strain NBRC 15520 (NR113764)
Bacillus thuringiensis strain BSPOCC	KX371253	1384	99	*Bacillus thuringiensis* strain ATCC 10792 (NR114581)
Bacillus cereus strain BSP	KX371254	1370	99	*Bacillus cereus* strain ATCC 14579 (NR074540)

Quantitaive degradation potential of the isolates

The degradation potential of all the isolates was quantified by HPLC and UPLC. The calibration curves obtained for the four hydrocarbons are given below based on which the residual concentration and % degradation was calculated. *B. cereus* and *B. thuringiensis* were most potent in degrading naphthalene and acenapthene followed by their ability to degrade benzene and toluene in the first 10 days of incubation. *B. cereus* degraded 94.6 % naphthalene, 92.9 % acenapthene, 66.1 % benzene and 58 % toluene. *B. thuringiensis* degraded 95.5 % naphthalene, 80.6 % acenapthene, 73.1 % benzene and 41.4 % toluene. Results show that *A. migulans* BROD was not able to degrade acenapthene in the first 10 days with 3.1 % degradation but degraded

naphthalene, benzene and toluene at 97.7 %, 69.9 % and 85.3 % respectively in the first 10 days. *A. migulans* KTPP on the other hand was able to degrade 1.7 % toluene and 40.1 % toluene in intial 10 days of incubation. *B. aneurinilyticus* RSP also showed very less activity when treated with toluene and acenapthene at 10.9 % and 26.2 % degradation respectively.Increasing the days of incubation slowly increased the bacterial growth in all the bacterial cultures which reveals in the percentage degradation for 20, 30 and 40 days. All the bacterial cultures when treated with 0.1 % benzene showed 100 % degradation in 30 and 40 days of incubation. In case of toluene treatment the degradation was enhanced after 10 days and reached 100 % in 20 days. Degradation of naphthalene reached at a maximum of 99.4 % for *A. migulans* BROD, *A. migulans* KTPP and *B. aneurinilyticus* after 40 days of incubation. Highest degradation (99.9 %) was observed when *B. thuringiensis* was treated with acenapthene during 40 days of incubation. All the bacterial isolates showed maximum activity when treated with naphthalene as the % degradation reached above 90 % in the first 10 days itself.

Heavy metal analysis of soil sediments by FAAS

Results show that the cadmium concentration in BSPOCC (0.75 ppm), KTPP (1.35 ppm), APR (0.98 ppm) and RSP (0.82 ppm) was slightly more than the permissible limits. Chromium concentration at 113.1 ppm was also slightly higher in sample BSP. The concentration of zinc was found to be more than permissible limits in samples SIPU (270 ppm) and KTPP (480 ppm). Concentrations of copper was high and that of mercury very high in all the seven samples.

Heavy metal resistance of identified bacterial species

Maximum tolerance was shown by *Bacillus thuringiensis* BSPOCC and *Aneurinibacillus migulanus* BROD against antimony (15 mM) and least tolerance was shown by four bacterial species except *B. cereus* BSP with MTC values of 0 mM for cadmium salts. *B. cereus* showed least tolerance against Hg^{2+} (0.25 mM) but was able to tolerate 10 mM of Mn^{2+} . The results for *B. thuringiensis* showed maximum tolerance for antimony (15 mM) and least tolerance for Cadmium salt (No microbial growth was observed). *A. migulans* BROD resisted the antimony concentration as high as 15 m M and also showed good resistence against Mn (10 mM). *A. migulans* KTPP showed maximum activity against Mn (8 mM) whereas *A. aneurinilyticus*

resisted 6 Mm of Pd, Zn, Mn and Sb.

Antibiotic resistance of strains

All the identified bacterial species showed resistance against penicillin G and cefuroxime and were susceptible to impenem, amoxyclav and tigecycline. B. cereus showed resistance or intermediate resistance against 6 out of 9 antibiotics and the other four (*B. thuringiensis* BSPOCC, *A. migulans* BROD, *A. migulans* KTPP and *A. aneurinilyticus* RSP) were resistant only to 2 out of 9 antibiotics used.

Discussion

In this study we aimed to obtain a strain collection of hydrocarbonoclastic bacteria, with an outstanding ability to degrade hydrocarbon pollutants and to resist elevated heavy metal concentrations at the same time, for further use in the development of a microbial soil inoculants for bioaugmentation purposes. Total ten PHC contaminated soil samples were collected from various locations in Chhattisgarh, India. To check the effect of contamination on different properties of soil, physicochemical analysis of the collected soil samples and one uncontaminated agricultural soil was carried out. PHC pollution exerts adverse effects on soil conditions, microorganisms and plants (Uche et al., 2011), leads to deterioration of soil structure, loss of organic matter contents, loss of soil mineral nutrients such as sodium, calcium, magnesium, nitrogen and sulphate, phosphate and nitrate (Akubugwo et al., 2009). There was no significant change in the pH of the contaminated and uncontaminated soil. When compared with pH of uncontaminated soil which was 7.7 the most alkaline were the soil samples from KTPP, RSP and BSP with pH values of 8.5. These results correlate with the findings of Margarita et al., (2014) and Efsun et al., (2015). All the samples were found to be slightly alkaline which is efficient for bioremediation (Vidali, 2001). As expected due to hydrocarbons from the petroleum, the organic carbon content in all the contaminated soil samples was significantly higher than normal soil. The nitrate and phosphate content of the soil samples was less than that of normal soil which has been reported by (Ahamefule et al., 2014). Lower concentration of nitrate and phosphate have been reported as limiting factors for the growth of microorganisms in PHC polluted environments (Rahman et al., 2002). The moisture content which determines the

extent of water retention and aeration in the soil was also less in PHC contaminated soils as that of normal soil. These two properties are important for the growth of biotic components in the soil. Presence of PHC in the soil increases the soil hydrophobicity (Khamehchiyan et al., 2007, Bennett et al., 1993; Roy et al., 1999), reducing the water holding capacity of the soil (Osuji and Nwoye, 2007). Bundy et al. (2002) have also reported that nutrient balance (C and N), pH and moisture content of soil were usually affected as a result of contamination by hydrocarbons. The altered physico-chemical properties of PHC contaminated soil makes it unfit for the growth of agricultural crops as well as the normal soil flora. Anoliefo and Vwioko (1995) observed that oil in soil created unsatisfactory conditions for plant growth, probably due to insufficient aeration of the soil. The soil temperature were slightly higher because of the components present in oil which absorbs light of both the visible and UV range (Yu et. al.,2006). Moreover the contaminants in soil form a dark coating which increases the subsurface soil temperature (Balk et. al., 2002).

Identification results of isolated hydrocarbon degrading strains suggested the dominance of the representatives of the Bacillus (Firmicutes) in all the samples. There have been fewer reports on the roles of *Bacillus cereus, Bacillus thuringiensis, Aneurinibacillus migulans and Aneurinibacillus aneurinilyticus* in hydrocarbon bioremediation although there are several reports of bioremediation of pollutants by the action of Bacillus sp. occurring in extreme environments. Sorkhoh et al. (1993) isolated 368 isolates belonging to the genus Bacillus from desert samples. In addition, Annweiller and co-workers (2000) described a *B. thermoleovorans* that degrades naphthalene at 60˚C. More recently, Ijah and Antai (2003) reported Bacillus sp. being the predominant isolates of all the hydrocarbon utilizing bacteria characterized from highly polluted soil samples. Nonetheless, the capacities that have been proven include survival on individual aliphatic and aromatic hydrocarbons (Verma et al. 2006; Ghazali et al. 2004; Das and Mukherjee, 2007), degradation of crude oil as well as oily sludge (Ijah and Antai, 2003).

The MPN count was always higher for heterotophic bacteria which were supplied with glucose as a sole source of carbon when compared to fuel degrader bacteria which were supplied with either benzene, toluene, naphthalene and acenapthene as sole source of carbon. MPN/gm was significantly lower in soil sediments as compared to the whole soil as most of the microbes were removed during the process of soil sedimentation. The presence of hydrocarbon further

decreased the MPN/gm values in all soil samples. Nevertheless, microbial populations can be one of the measures for evaluating the level of petroleum contamination at these sites. The concentration of hydrocarbons is a major factor contributing to the number of microorganisms adapted to degrade that hydrocarbon. But other factors like availability of different nutrients and terminal electro acceptors are also responsible for proper microbial growth (Leahy and Colwell. 1990). Similar studies have been done in gasoline contaminated aquifiers in the artic (Braddock and McCarthy, 1996)

From the degradation abilities obtained by quantification of residual hydrocarbons by HPLC and UPLC it is clear that the identified bacterial species are able to detoxify the hydrocarbons and present a suitable candidature for bioremediation programs. Very less reports were available which emphasize on microbial bioremediation of benzene, toluene, naphthalene and acenapthene when treated with either of the four identified bacterial species. Dolanchapa et al. 2016 have reported the degradation of acenapthene using two isolated micro-organism *Bacillus sp. PD5*. There has been reports of acenapthene degradation by other bacterial species as reported by Selinofov et al. 1993 and Komatsu et al. 1993. Napthalene degradation by Bacillus strains have been reported by Silva et al. (2009b). Similarly Lidija et al. (2011) have reported the bioremediation of toluene and naphthalene by B. cereus including three other bacterial species. Anaerobic degradation of benzene has been most widely studied (Anderson et al. 1998; Chakraborty and Coates, 2005; Da Silva and Alvarez, 2007) but fewer studies have been done on aerobic biodegradation of benzene.

Heavy metal resistance of strains was tested in nutrient broth containing different concentrations (0.25, 0.5, 1.0, 2.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 mM) of $CdCl_2$, $CuSO_4.5H_2O$, $Pb(NO_3)_2$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, $C_8H_4K_2O_{12}Sb_2.3H_2O$, $SnCl_2.2H_2O$, $K_2Cr_2O_7$, $NiSO_4.6H_2O$ $HgCl_2$, $FeSO_4.7H_2O$ (HiMedia). Amongst the tested heavy metals Pb^{2+} , Cu^{2+} , $Mn^{2+,}$ Sb^{3+}, Sn^{2+} and Zn^{2+} were the most tolerated by the tested strains, and the upper limits of tolerance were rallied around a wide range of values (Sn^{2+} - 4 mM to Sb^{3+} - 15 mM). As the concentrations of some metals like Zn, Cu, Cd, Cr and Hg were found higher than the permissible limits, it can be said that these bacterial species have developed a mechanism to tolerate abnormal metal concentrations. The presence of Cd^{2+} was very toxic to all the isolates except B. cereus BSP which tolerated 1mM concentration. The presence of Hg^{2+} had an inhibitory effect on all the

isolates with tolerance limit of only 0.25 mM inspite of high concentrations found in FAAS analysis. This is in contrast with other findings that a microbial population living in heavy metal polluted site should have the ability to tolerate high metal concentrations (Chiu et al., 2007). Outstanding ability of *Bacillus* species in remediation of heavy metals has been demonstrated in various studies (Ferdag et al., 2011; Ersoy et al., 2009; Othman and Thoufeek 2015; Yogendra et al., 2013). Among the various possibilities of metal–microbe interactions (viz. bioaccumulation/ biosorption, enzymatic/extracellular transformations, etc.) that determine environmental fate of toxic cations, metal sequestration by bacterial cells is of great importance for the development of microbe based remediation strategies (Lloyd and Macaskie, 2000). *B. cereus* showed resistance / intermediate resistance against 6 of the 9 antibiotics whereas rest of the isolates showed resistance only against 2 antibiotics (Penicillin and Cefuroxime). Multiple resistance against heavy metals and antibiotics of *B. cereus* isolates has also been reported by Singh et al. (2010). Since several genes responsible for degradation of aromatics and for heavy metal/antibiotic resistances are located on plasmids which are key vectors of horizontal gene transfer, the members of the bacterial community gained opportunity to expand their chromosome encoded resistance and catabolic potential with those encoded on plasmids. This phenomenon may explain the strong correlation among hydrocarbon degradation ability and heavy metal/antibiotic tolerance among strains. In case of strains isolated from the solely PHC impacted sample the lack of correlation among foregoing capabilities might be linked to the lack or low rate of transmission of mobile genetic elements. In the absence of a strong driving force (e.g. presence of heavy metals or antibiotics) the endogenous micro biota is not actuated for the exchange of resistance carrying plasmids. Our findings justify the above statement that in the absence of antibiotic driving force in the environment around soil sample collected caused the partial gene transfer for heavy metal resistance only and not for antibiotic resistance. Furthermore, as several biodegradative pathways are located also on mobile genetic elements, a long term exposure to heavy metals/antibiotics may be linked to the widespread distribution of biodegradative capabilities as well (Roy et al., 2002). Nevertheless, the lack or complete/partial loss of these transmissible genetic segments may lead to the reduced degradative functions, as well as to the loss of multiple resistances (Amábile-Cuevas et al., 1991; Marqués and Ramos, 1993).

SUMMARY AND CONCLUSION

This thesis presents a study of the bacteria bioremediation characters and their heavy metal and antibiotic tolerance from the petroleum contaminated soil located in industrialized areas of Chhattisgarh, India. Soil sediments were characterized by FTIR for the presence of aromatic and aliphatic compounds and were also characterized for heavy metal contamination by FAAS. Further soil sediments were analyzed for heterotrophic and fuel degrader counts by ELISA micro plate technique using tetrazolium violet as indicator for microbial respiration. Most potent strains characterized were predominantly from the Bacillus species which showed mixed resistance patterns against antibiotics and heavy metals. All the bacterial cultures when treated with 0.1 % benzene showed 100 % degradation in 30 and 40 days of incubation. In case of toluene treatment the degradation was enhanced after 10 days and reached 100 % in 20 days. Degradation of naphthalene reached at a maximum of 99.4 % for *A. migulans* BROD, *A. migulans* KTPP and *B. aneurinilyticus* after 40 days of incubation. Highest degradation (99.9 %) was observed when *B. thuringiensis* was treated with acenapthene during 40 days of incubation. All the bacterial isolates showed maximum activity when treated with naphthalene as the % degradation reached above 90 % in the first 10 days itself. *Bacillus cereus* strain BSP showed resistance against most heavy metals and also was the second most active bacteria to degrade the selected hydrocarbons. All the isolates except *Bacillus cereus* were resistance only to Penicillin G and Cefuroxime and sensitive to others which shows that the transfer of antibiotic resistance genes has not taken place.