An Investigation on Organic solvents tolerance, Heavy metals and Antibiotics resistance of *Bacillus oleronius* from Petroleum contaminated soil

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Abstract: The petroleum contaminated soil can be exploited as an ideal environment to investigate the tolerance and resistance mechanisms of bacteria against organic solvents and antimicrobials. In this study, *B. oleronius* was isolated from petroleum contaminated soil and characterized by morphological features, biochemical tests and 16S rRNA sequencing. The bacteria were explored for its tolerance and resistance to organic solvents, heavy metals and antibiotics respectively. Agglutination of O^- blood RBCs by *B. oleronius* was observed. Our results have conclusively proved that *B. oleronius* have shown moderate tolerance and resistance to the organic solvents, heavy metals and antibiotics tested. Hence it is likely that the bacterial isolates from petroleum contaminated site tend to show extensive adaptation to organic solvents, heavy metals and antibiotics.

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1. Introduction

Petroleum spill remains a huge problem by contaminating land and water, thus propose to be major environmental pollutants. Petroleum contaminated site contains toxic organic solvents such as benzene, toluene, xylene, cyclohexane, hexane, petroleum ether and heavy metals such as nickel, vanadium, copper, cadmium, lead, chromium, zinc, selenium and mercury with potential vulnerability to native microorganisms (Ismail and Beddri, 2009). The petroleum impacted soil consists of high concentration of organic solvents, heavy metals and are characterized by fluctuations in temperature, pH and salt concentrations. Hence the microorganisms surviving the petroleum stress tends to adapt by its ability to limit environmental yoke (resistance) and limit the harm caused by environmental stress (tolerance), which will enable them an ecological and evolutionary advantages to counteract heavy metals, antibiotics and organic solvent stress in contaminated site.

Bacteria utilize either one or collective of these mechanisms to tackle harsh environment such as transformation of harmful compounds to non toxic products, horizontal gene transfer of mobile DNA along with heavy metals and antibiotics resistant genes (Bezverbnaya *et al*., 2005), pigments to protect from multiple antibiotics and heavy metals (Hermansson *et al.*, 1987) and direct pumping out of antimicrobials & organic solvents by efflux pumps (Ramos *et al*., 2002). The presence of a high concentration of heavy metals and organic solvents in petroleum contaminated site serve as a selective environment favoring tolerant and resistant bacteria. There are few reports to show the overlapping relation between the heavy metals & antibiotic resistance of microbes (Davidson *et al*., 1999) and antibiotic resistance & organic solvent tolerance of bacteria (Kadavy *et al*., 2000).

Compared to non-pigmented bacteria, pigmented bacteria represent the predominant agents of hydrocarbon contaminated sites (Leahy *et al*., 1990) due to their innate property of tolerance/resistance adaptation in the extreme environments. Bacterial community such as *Dietzia* (von der Weid *et al.,* 2007), *Gordonia* (Shen *et al*., 2008), *Microbacterium testaceum* (Edward *et al*., 2011), *Pseudomonas*, *Arthrobacter*, *Alcaligenes*, *Corynebacterium*, *Flavobacterium*, *Achromobacter*, *Micrococcus* and *Nocardia* (Englert *et al*., 1993) has been identified and characterized from petroleum contaminated soil by culture dependent 16S rRNA gene sequences. *Bacillus oleronius* is an opportunistic pathogenic bacteria which causes acne rosacea in humans and found in the hindgut of the termite *Reticulitermes santonensis* (Kuhnigk *et al*., 1995) and human skin parasitic mite *Demodex folliculorum* (Lacey *et al*., 2007). *B. oleronius* has been reported to present in extreme environments such as hypersaline lake (Philips *et al*., 2012), estuary contaminated with heavy metals (Jose *et al*., 2011) and dairy forms by producing heat-resistant spores (Scheldeman *et al*., 2005).

The present study is aimed to isolate and characterize *B. oleronius* for their tolerance and resistance to organic solvents, heavy metals and antibiotics respectively. Additionally its ability to agglutinate blood RBCs was studied.

2. Materials and Methods

List of Chemicals

Heavy metal salts and antibiotics utilized for experiment were of analytical grade and procured from Himedia, India. Organic solvents such as cyclohexane, hexane, toluene, xylene and petroleum ether were bought from Merck, India. Muller-Hinton agar (MHA) and Luria Bertani (LB) media utilized for antimicrobial studies & bacterial culture studies were bought from Himedia, India respectively.

Isolation of Bacteria

The soil sample was collected from petroleum contaminated site and made into slurries by mixing with sterile distilled water, overlaid with petrol and kept unshaken for two days to concentrate the bacterial populations. The sample was serially diluted, plated on LB agar plates and incubated at 37°C. Colonies appeared after 2 to 3 days of incubation. A non-pigmented bacteria was isolated, pure cultured and stored at -20°C till further use.

Preliminary characterization of isolates

Morphological features of WP bacterial colonies such as form (circular, filamentous and irregular); elevation (flat, convex, and umbonate); margin (entire, undulate, erose and filamentous) and optical features (opaque, translucent, and transparent) were studied (Pelczar and Reid, 1958). Gram staining was done to find out the shape and gram character of the bacteria. Motility tests were done by inoculating the bacteria into semi-solid LB agar medium and observed for the migration pattern of bacteria from the point of inoculation.

Biochemical characterization

The biochemical characterization of the isolated bacteria was studied by methyl red test, Voges-proskauer test, catalase test, Mac conkey agar test and citrate utilization test. The bacteria were identified by Bergey's Manual of Determinative Bacteriology (7^{th} Edition) as a reference.

DNA extraction and 16S rDNA amplification

Genomic DNA was extracted using spin column based Genomic DNA Purification Kit (Chromous Biotech, India) according to manufacturer's instructions. For the 16S rRNA amplification of *B. oleronius,* universal primers were used. 50 µl of PCR master mix contained 25pm each of 16F27 (5′-CCA GAG TTT GAT CMT GGC TCA G-3′) forward and 16R1525 (5′-TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC-3′) reverse primers, 10 mM of dNTPs, 1X PCR Buffer, 1U Taq polymerase, 2mM Mg^{++}, 1 µl (100-200 ng) template DNA, sterile milli Q water. Phylogenetic tree for *B. oleronius* was constructed using the neighbor-joining method by MEGA 5.0 program (Tamura *et al*., 2011).

ORGANIC SOLVENT TOLERANCE ASSAY

Solid medium overlay assay

The bacterial isolate was grown to late logarithmic phase and diluted to a concentration of approximately10^7 cfu/mL. A 5µL aliquot of the bacterial suspension was spotted onto LB agar plates and air dried. Xylene, Toluene, Cyclohexane, Petroleum ether and hexane solvents were added to individual agar plates to a depth of 2 to 3 mm respectively. Plates were sealed with parafilm to prevent evaporation of solvents and incubated at 30°C for 24 h. Later the solvents were drained out from agar plates, incubated at 30°C for 24h and observed for the growth pattern of bacteria compared to the control plates (Asako *et al*., 1997). Plating was done in triplicates and solvent tolerance was measured as a function of bacterial growth. Growth was recorded as confluent growth, single colony growth, or no growth after 24 h.

HEAVY METAL RESISTANCE ASSAY

Well diffusion assay

Well diffusion assay was done to screen the heavy metals resistance patterns of bacterial isolate. MHA plates were prepared and 100µl of bacterial culture was surface swathed with the L-Rod aseptically. The bacterial cultures were allowed to dry and 0.5cm wells were prepared with the gel puncher. 20µl of different concentrations (200 ng/ml, 500 ng/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml and 80 µg/ml) of heavy metals were added to the wells and incubated at 37°C for 24 hrs. The diameter of the inhibition zone around the wells was measured in millimeters.

ANTIBIOTIC RESISTANCE ASSAY

Inhibition zone assay

Inhibition zone assay was performed to screen for antibiotic resistance patterns of bacterial isolate as per the protocol of Chandy, 1999 with slight modifications. 100µl of bacterial culture was evenly spread on the MHA plates and air dried. The respective wells were bored using gel puncher and 20µl of varying concentrations of antibiotics

(200ng/ml, 500ng/ml, 1µg/ml, 5µg/ml, 10µg/ml, 20µg/ml, 40µg/ml, and 80µg/ml) was added. The agar plates were incubated at 37°C for 24 hrs. The diameter of the inhibition zone was measured in millimeters. Each test was conducted three times with triplicates each.

Determination of human RBCs agglutination

The bacterial isolate was cultured in LB broth overnight and centrifuged (5000 rpm for 10 min). The pellets were washed with Phosphate buffer saline (PBS) to remove the cellular debris and centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the bacterial pellet was dissolved in 800µl of PBS to make a thick suspension of cells. Five different human bloods (A_1^+, A_2B^+, B^+, B^- and O^-) were collected and immediately centrifuged. The serum was discarded and the RBCs were washed with the PBS till the red colour fade. Equal volume of PBS compared to discarded serum was added to RBCs and considered as 100% RBCs solution. 4% RBCs were prepared. Serial dilutions of bacterial suspensions were done in V shaped microtiter plates and to that, equal volume (50µl) of different blood RBCs were added respectively and gently mixed. The suspension was kept at room temperature for 30 minutes. The agglutination of RBCs by bacterial culture was noted.

Statistical analysis

Statistical analysis was performed using SPSS software (version 17.0). Statistical analysis included mean ± Standard Deviation (SD), ANOVA and Student's *t* test. Results were considered significant at $P < 0.05$.

3. Results

Morphological and biochemical analysis

The soil sample was collected from contaminated sites in Tamilnadu. Non-pigmented WP bacterial strain has been isolated from contaminated soil. Morphological and biochemical analysis of isolate WP were done. The bacteria has formed cream coloured colony with circular shape, mucoid nature, flat elevation with smooth margin. It was found to be gram negative bacteria which showed negative reactions for methyl red and Voges-Proskauer test indicate its inability to convert glucose to ethanol or butanediol and glucose to acetoin respectively. No growth was observed in Mac conkey agar medium indicating that the bacteria were susceptible for bile salts and not belongs to enterobactericeae family. Positive reactions were observed for catalase enzyme and citrate utilization emphasizes the aerobic respiration nature and conversion of citrate into oxaloacetate respectively. The bacteria were found to be non-motile as there was no observation of bacterial movement from the site of inoculation. Presence of spore was observed in bacterial isolates when examined under microscope. Based on morphological and biochemical characterization, the bacterial isolate was identified up to *Bacillus* genus level. The morphological and biochemical test results are provided in Table 1.

Table 1. Morphological and Biochemical characteristics of *B. oleronius*

Characteristics	***A. oleronius***
Shape	Circular
Pigmentation	White
Opacity	Opaque
Texture	Mucoid
Spreading nature	Non-spreading
Elevation	Flat
Margin	Entire, smooth
Gram Staining	Gm^{-ve}
Motility Test	Non-motile
Spore formation	Positive
Methyl red	Negative
Voges-Proskauer	Negative
Catalase	Positive
Citrate utilization	Positive
Mac conkey agar	Negative

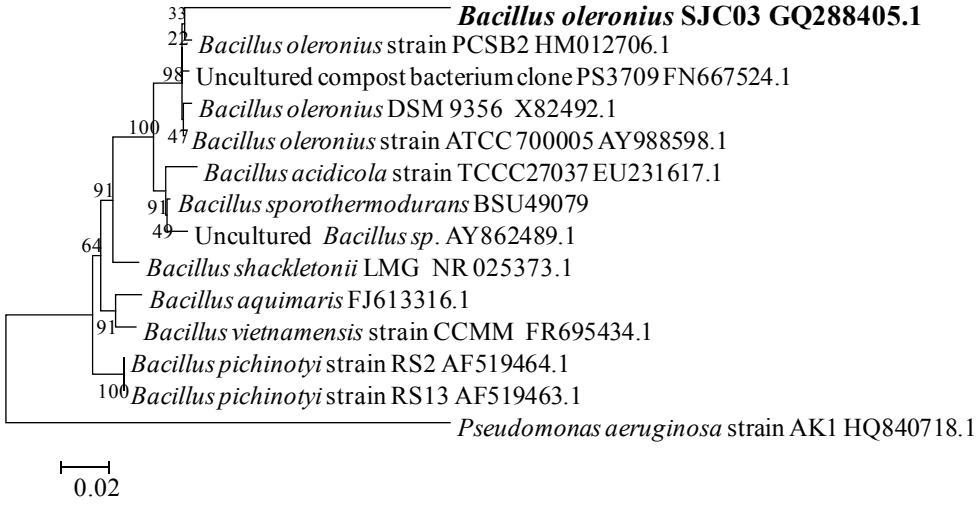
Genomic DNA extraction, Molecular detection and phylogenetic analysis of isolates

Extracted genomic DNA was found to be free from protein, RNA contaminants and of good quality as evidenced by agar gel electrophoresis. The PCR parameters were optimized for the maximum amplification of 16S ribosomal RNA gene. PCR includes initial denaturation at 95°C - 5 min., 35 cycles of denaturation at 95°C for 1 min., annealing at 55°C for 1 min., elongation at 72°C for 1 min. and final extension at 72°C for 3 min. 1.5 kb amplicons were generated. Size of PCR amplicon was estimated by using DNA marker (100 base-pair ladder). The amplicons were separated on 1.5% agarose gel and photographed using Total Lab Gel Documentation System, England. Finally the amplicons were extracted and purified using GeNeiPure™ Gel Extraction kit (Bangalore Genei, India) and sequenced commercially using AB DNA sequencer (Chromous Biotech, Bangalore, India). Comparing the sequence of the 16S rDNA gene of our isolate with the sequences in GenBank by BLAST revealed that our 16S rDNA sequence (GQ288405) is similar to *B. oleronius* (AY988598.1) with 99% similarity. Nucleotide sequences of partial 16S rRNA genes

were deposited with GenBank under the following accession number: GQ288405**.**

A neighbor-joining tree based on 16S rDNA sequences showed that our isolate occupies specific clade with other *Bacillus* species. Figure 1 represents the 16S rDNA phylogenetic tree, showing the relationship between our *B. oleronius* strain SJC03 and known genera forming distinct clusters. Phylogenetic tree has revealed that our isolate has closely related to *B. oleronius* PCSB2 (HM012706) and deviates evolutionarily much from other *Bacillus* species. Kimura-2 parameter was used as the nucleotide substitution model. The bootstrap values (%) presented at the branches was calculated from 1000 replications. *Pseudomonas aeruginosa* strain AK1 (HQ840718.1) was used as an out-group. Scale bar indicates 0.02 substitutions per nucleotide position.

Figure 1. 16S rDNA phylogenetic tree, showing the relationship between SJC03 strain and other *B. oleronius* species.



Organic solvent tolerance assay

B. oleronius has shown the moderate tolerance for the organic solvents tested (Table 2). Single colony growth was observed in LB agar plates immersed with hexane and petroleum ether. No growth was observed in toluene, xylene and cyclohexane treated plates. Confluent growth was observed in control plates.

Heavy metal resistance

B oleronius were explored for resistance to 9 different heavy metals (Table 2). It has showed high resistance to potassium dichromate, cobaltous chloride, Copper chloride, Ferrous sulphate, Lead acetate and Nickel chloride as there were no zone of inhibition around the wells observed. Contrary to the above results, least resistance was observed for mercury followed by cadmium chloride and silver nitrate. These results have shown that *B. oleronius* possess varying resistance and susceptibility towards different heavy metals.

Antibiotic resistance pattern

Three antibiotic resistance patterns of *B. oleronius* were evaluated (Table 2). *B. oleronius* has showed significant susceptibility for rifampicin followed by chloramphenicol with high resistance for ampicillin concentrations tested. Increasing the concentration of antibiotics results in the substantial increment of bacterial susceptibility towards antibiotics (Plate 1).

Agglutination of human RBCs

The agglutination was observed only in O^- RBCs, but absent in five other blood groups.

Plate 1. Antibiotic resistant pattern of *B. oleronius*

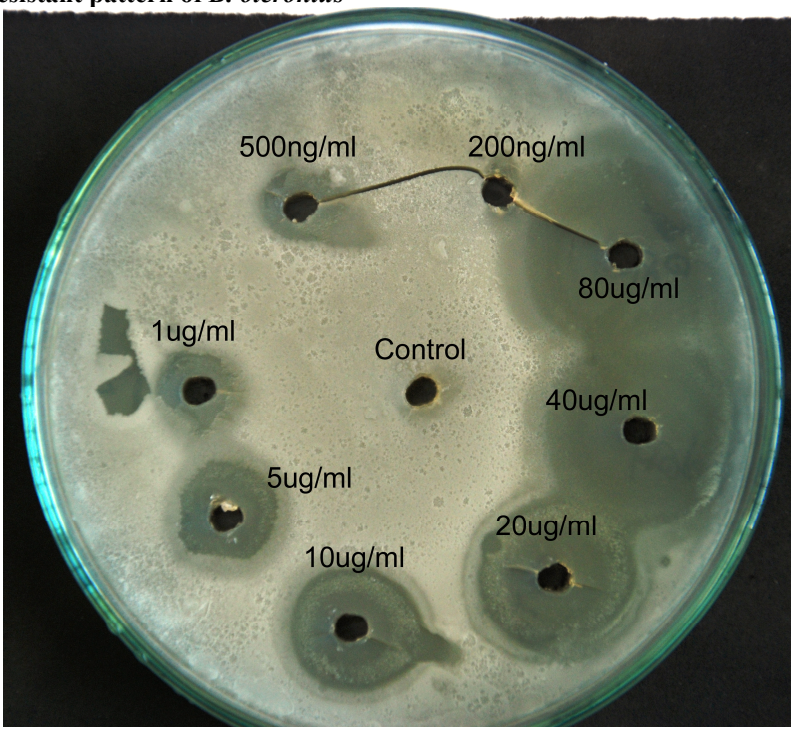


Table 2. Tolerance and Resistance of *B. oleronius* to Organic solvents, Heavy metals and antibiotics

Organic solvents	**Control**	**Hexane**	**Toluene**	**Xylene**	**Petroleum ether**	**Cyclohexane**
Growth	Confluent	Single colony	No growth	No growth	Single colony	No growth

Heavy metals	**Zone of clearance (mm)**							
	200ng/ml	**500ng/ml**	**1µg/ml**	**5 µg/ml**	**10 µg/ml**	**20 µg/ml**	**40 µg/ml**	**80 µg/ml**
Mercury chloride	--	--	--	--	8.7±0.115	12.3±0.11	13.3±0.05	16.7±0.05
Silver nitrate	--	--	--	--	--	--	6.3±0.05	9.3+0.05
Potassium dichromate	--	--	--	--	--	--	--	--
Cadmium chloride	--	--	--	--	--	6.5±0.05	8±0.1	10.3±0.15
Cobaltous chloride	--	--	--	--	--	--	--	--
Copper chloride	--	--	--	--	--	--	--	--
Ferrous sulphate	--	--	--	--	--	--	--	--
Lead acetate	--	--	--	--	--	--	--	--
Nickel chloride	--	--	--	--	--	--	--	--
Antibiotics	**Zone of clearance (mm)**							
	200ng/ml	**500ng/ml**	**1µg/ml**	**5 µg/ml**	**10 µg/ml**	**20 µg/ml**	**40 µg/ml**	**80 µg/ml**
Rifampicin	11±0.1	12.6±0.05	14.6±0.05	19.6±0.05	24.3±0.05	26.3±0.05	31±0.05	32.3±0.05
Chloramphenicol	--	--	--	12±0.1	14.6±0.05	21.6±0.15	23.6±0.05	24.3±0.05
Ampicillin	--	--	--	--	--	--	--	--

*Mean±SD

4. Discussion

Gram negative bacteria exhibit tolerance and resistance to organic solvents, heavy metals and antibiotics by altering its plasma membrane lipid content and efflux pumps to siphon out toxic organic solvents out of the cell (Ramos *et al*., 2002). Since *B. oleronius* belongs to gram negative bacteria, it has been anticipated that the isolate may have either

utilized either one or collective of the above mentioned mechanisms for its survival in solvent rich petroleum contaminated site. Gram negative bacteria such as *Pseudomonas* and *Aeromonas hydrophila* (Lazaroaie, 2009; Liu *et al*., 2011) has been reported to show high organic solvent tolerance compared to gram-positive *Enterococcus, Staphylococcus* and *Rhodococcus* strains (Nielsen *et al*., 2005; Paje *et al*., 1997). *B. oleronius* have shown the tolerance against two organic solvents, moderate resistance against three heavy metals and highly susceptible for two classes of antibiotics. This may be due to the selective emergence of isolates from petroleum contaminated environment in the presence of high concentration of heavy metals and toxic solvents (Nakahara *et al*., 1977). The bacteria which subjected to organic solvents can exhibit high antibiotic resistance and heavy metal resistance due to the siphon out of antimicrobials by efflux pumps (Nielsen *et al*., 2005, Godoy *et al*., 2001). Verma *et al*., 2001 has reported the close linkage between heavy metal resistant and antibiotic resistant genes in plasmids results in the increased microbial resistance for antimicrobials. Bacteria have reported to spread and transfer R – factors when it facade heavy metals stress (Bhattacherjee *et al*., 1988). All the reports are consistent with our results and showed the correlation between organic solvent tolerance, heavy metals resistance and antibiotics resistance of our bacterial isolate. Bacteria possess pili/fimbriae can agglutinate red blood cells and other bacteria via multiple adhesin–receptor interactions. Bacteria agglutinating red blood cells could also agglutinate other bacteria. Hence this could be a defensive strategy employed by the bacteria to protect itself from the environmental stress or immune system by forming a mesh of bacteria or RBCs around them respectively. Few bacteria have been reported to agglutinate human RBCs by fimbriae mediated e.g. *Serratia marescens* (Martincic *et al*., 2008) and non fimbriae mediated e.g. *Escherichia coli* (Hoschutzky *et al*., 1989). The organic solvents, heavy metals and antibiotics utilized for our studies were hydrophobic in nature. Efflux pumps siphon out hydrophobic substances efficiently than hydrophilic compounds. Hence we speculate that the efflux pumps can give resistance and tolerance to *B. oleronius* to withstand the extreme stress. It is therefore likely that if bacteria subjected to either organic solvents/heavy metals/antibiotics stress, the selective pressure will activate the whole set of resistances in microbes.

5. Conclusion

Petroleum contaminated soil is a stressful environment which insist the bacterial community to adapt to survive in harsh environment. The contaminated site is an ideal source for the study of bacterial tolerance and resistance to antimicrobials. The present study revealed that the bacteria can show multiple tolerance and resistance mechanisms against organic solvents, heavy metals and antibiotics. Future perspectives include the evaluation of *B. oleronius* isolate as an extremophiles and finding out the role of efflux pumps in organizing tolerance/resistance mechanisms to the bacteria. In conclusion, *B. oleronius* could be an ideal candidate to be utilized in solvent industries and heavy metal removal from effluents.

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