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Role of Efflux Pumps in Affording Organic Solvent Tolerance to Bacterial Strains from Petroleum Contaminated Soil

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Abstract: Petroleum contaminated site has a high concentration of organic solvents and heavy metals. Bacteria surviving in the contaminated site will implement efflux systems to evade from toxic organic solvents. In this study, an attempt was taken to perceive the relation between efflux pump activity and organic solvent tolerance of bacteria. *B. oleronius* isolated from petroleum contaminated site was utilized to investigate the role of efflux pump in organic solvent tolerance. The viability of cells was significantly reduced in broth supplemented with test solvents compared to untreated bacteria. Rhodamine B accumulation and efflux studies divulged that the bacterial isolate has greatly utilized the efflux pump system to accumulate and siphon out the dye. The intrinsic correlation between solvent tolerance and efflux pumps was studied by treating the bacterial isolates with efflux pump inhibitors, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 2,4 Dintrophenol (DNP) in the presence and absence of organic solvents. Compared to the control, treated isolates have failed to grow and showed different growth pattern indicating the critical role of efflux pumps in solvent tolerance. Antimicrobials potentiation of solvent tolerance on *B. oleronius* has showed significant changes in the growth pattern and presaged the activation of efflux pump which in turn activated the solvent tolerance mechanisms. These results showed that bacteria employ a wide array of efflux systems to tackle the high concentration of solvents in the contaminated site.

Key words: Petroleum · Efflux Pump · Organic Solvents · Potentiation · Dinitrophenol

INTRODUCTION

Petroleum contamination occurs by the substantial release of hydrocarbon effluents into the environment [1], thus generating bio-hazards for the survival of microbial communities [2]. Petroleum hydrocarbons are versatile muddle of saturates, aromatic organic solvents (toluene xylene, styrene, benzene, cyclohexane, petroleum ether and hexane), asphaltenes (phenols, fatty acids, ketones, esters and porphyrins), resins (pyridines, quinolines, carbazoles, sulfoxides and amides) and heavy metals such as vanadium and nickel [3, 4].

Petroleum slosh alters the physical, chemical and biological conditions of soil and generates the significant predicament for the native microorganisms due to the high concentration of organic solvents and heavy metals. Most of the organic solvents are extremely lethal to microorganisms by affecting the rigidity and stability of plasma membrane thus results in the leaking of intracellular contents and eventual death of microorganisms [5, 6]. The toxic nature of the organic solvents is due to their Log P_{OW} values i.e. partition coefficient between n-octanol and water. An organic solvent with low Log P_{OW} value tends to exhibit lethal effects on microorganisms than solvents with high Log P_{OW}.

Microorganisms that can be able to tolerate high concentration of organic solvents (10-50%) are solvent tolerant and are considered as extremophiles. Bacteria have developed various metabolic, biochemical and physiological protective mechanisms to tackle the lethal effects of organic solvents such as biotransformation of solvents into non toxic byproducts [5], membrane rigidification [7], compartmentalized vesicles to remove solvents [8] and the extrusion of organic solvents via energy dependant process [9].

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In order to sluice out toxic organic solvents, bacteria prefer efficient and active efflux systems than other physical barriers [10, 11]. Gram positive bacteria such as *Rhodococcus* and *Bacillus* have been reported to possess solvent tolerance [12] and utilize the efflux pump activity to retain solvent tolerance as Gram negative bacteria [5]. Bacteria such as *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* and *Salmonella enterica* has been reported to extensively use efflux pumps for its enhanced multi drug resistance [13-15]. There were few reports published earlier concerning RND (Resistance-Nodulation-Cell division) efflux pumps [16], MexAB-OprM, MexCD-OprJ and MexEF-OprN efflux system [10] and AcrAB-TolC efflux pump [17] in the pumping out of toxic solvents from the bacterial cells. The present investigation aimed to study the role of efflux pumps in providing the solvent tolerance to *B. oleronius* isolated from petroleum contaminated site.

MATERIALS AND METHODS

List of Chemicals: Analytical grade antibiotics (Geneticin, Hygromycin), Heavy metals (Potassium dichromate, Cadmium chloride), detergents (Sodium dodecyl sulphate (SDS), Cetyl trimethyl ammonium bromide (CTAB), Luria Bertani (LB) media, Efflux pump inhibitors (EPI) such as CCCP and DNP were procured from Himedia, India. Organic solvents (cyclohexane, hexane, toluene, xylene and petroleum ether) were bought from Merck, India.

Isolation of Bacteria: The soil sample was collected from petroleum contaminated site, mixed in sterile distilled water to form a thick paste and overlaid with petrol. The sample was serially diluted, plated on LB agar plates and incubated at 37°C for 48h. The suspected colonies were isolated, pure cultured and stored at -20°C till further use.

DNA Extraction and Amplification of 16S Ribosomal DNA: Genomic DNA was extracted using the commercial Genomic DNA Purification Kit (Chromous Biotech, India) according to manufacturer's instructions. For the 16S rRNA amplification of *B. oleronius,* universal primers were used. PCR amplification was performed in 50 µl containing 1U of Taq DNA polymerase (Fermentas, Canada), 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, 200 ìmol/liter each of PCR grade dNTPs, 1X PCR buffer, 2mM Mg^{++}, 1 µl (100-200 ng) extracted DNA and sterile Milli Q water. PCR reactions was performed in a thermocycler (Eppendorf, Germany) with the following conditions: denaturation at 95°C (5 min), followed by 30 cycles of denaturation at 95°C (60 sec), annealing at 55°C (60 sec), extension at 72°C (60 sec) and final extension step at 72°C (5 min). 25µl of PCR amplicons was visualized in 1.2% agarose gel stained with ethidium bromide, photographed and analyzed using Total Lab Gel Documentation software, England. Finally the amplicons were purified from the agarose gel using GeNeiPure™ Gel Extraction kit (Bangalore Genei, India) and sequenced commercially using AB DNA sequencer (Chromous Biotech, Bangalore, India). BLAST analysis utilizing FASTA search option of our obtained 16S rRNA sequence with deposited DNA sequences of bacteria in GenBank has rummaged the close evolutionary relatives. Phylogenetic tree for *B. oleronius* was constructed using the neighbor-joining method by MEGA 5.0 program [18]. Kimura-2 parameter was used as the nucleotide substitution model. The bootstrap values (%) presented at the branches was calculated from 1000 replications. *Pseudomonas geniculata AFB7* (GU971729.1) was used as an out-group. Scale bar indicates 0.02 substitutions per nucleotide position.

Organic Solvent Tolerance Assay: Log phase bacterial cultures (10^7 cells/ml) were blended with 20% (v/v) test solvents (Hexane, Cyclohexane, Toluene and Petroleum ether) respectively and incubated for 24 h at 30°C. Flasks were tightly sealed to prevent the evaporation of solvents. After incubation, the bacterial suspension were diluted and plated on LB agar medium and the viable cells tolerant to solvents were counted. The experiment was done in triplicates [19].

Preliminary Efflux Pump Assay

Accretion of Rhodamine B dye: By the bacterial isolate in the presence of organic solvents was done according to the protocol of Lazaroaie [20] with slight modifications. Bacterial cells were spotted on solid LB agar medium (control) and LB agar medium supplemented with 100 $\mu g \bullet ml^{-1}$ rhodamine B. The plates were examined under UV light for the accrual of rhodamine B in bacterial cells after 24 h of incubation at 28°C.

Efflux Pumps Inhibitors and Organic Solvent Tolerance: The inherent property of efflux pumps related to solvent tolerance of bacterial isolates was studied by pre-treating the bacteria with CCCP & 2,4 DNP [lower than MIC value i.e. 80µg/ml]. The effects of EPI on bacterial growth, efflux pumps activity and organic solvent tolerance was tested by the solid medium overlay assay with slight

modifications [21]. 5 µl of EPI treated and control bacterial suspension was spotted on LB agar plates and air dried respectively. Medium was covered by test solvents to the depth of 2 to 3 mm and the plates were sealed with Para film to prevent vaporization of solvents. After 48h of incubation at 30°C, bacterial growth was recorded as confluent growth (C), single colony growth (S), or no growth (NG). Plating was done in triplicates.

Antibiotics, Heavy Metals and Detergents Potentiation of Solvent Tolerance: The antibiotics, heavy metals and detergents potentiation of solvent tolerance was performed as follows: 10 µl of overnight bacterial culture was inoculated into 180µl of LB broth supplemented with the required concentrations of heavy metals, antibiotics and detergents in flat bottom microtitre plates [Geneticin (500ng/ml), Hygromycin (10µg/ml), Potassium dichromate (20µg/ml), Cadmium chloride (20µg/ml), SDS (10µg/ml) and CTAB (5µg/ml)] which were less than minimum inhibitory concentration values (data not shown) and incubated for 6h at 37°C. The concentration of antimicrobials was carefully selected to inactivate the efflux pumps and not to kill the bacterial cells. After incubation, 5µl of antimicrobials treated and control bacterial suspension were spotted on LB agar plates and air dried. Then the plates were overlaid with test organic solvents and incubated for 24h at 30°C. The bacterial growth was scored as no growth (NG), single colonies (SC), or confluent growth (C).

Rhodamine B Accumulation Assay: To determine the efflux pumps activity, the accumulation assay of Rhodamine B was performed.

10 ml of early Log phase culture was centrifuged and the supernatant was discarded. The pellets were washed twice with phosphate buffer saline (PBS) to remove the cell debris and other contaminants. Finally 10ml of PBS was added to the pellet and vortexed well. 19.2µl of Rhodamine B was taken from stock solution (10mg/1ml), added to the 10ml of bacterial suspension and kept in the rotary shaker at 150 rpm. At every 15 min interval, 1ml of culture was taken and centrifuged at 10,000 rpm for 5 min. 100µl of cell free supernatant was taken and OD value was measured at 545 nm.

Rhodamine B Efflux Assay: To confirm the efflux pumps activity, the efflux assay was performed using Rhodamine B with slight modifications [22]. 10 ml of early log phase culture was centrifuged and the supernatant was discarded. The pellets were washed twice and suspended in 10ml of PBS with increasing concentration of glucose (10, 50, 100 and 1000mM). 19.2µl of Rhodamine B stock solution (10mg/1ml) was added to the culture and shaken at 150 rpm for 1h. At every 15 min regular interval, 1ml of culture was taken and centrifuged at 10,000 rpm for 5 min. 100µl of supernatant was taken and OD values were observed at 545 nm.

RESULTS

Isolation and Identification of Bacteria: Non-pigmented, transparent, mucoid and smooth margin bacteria were isolated from soil sample. PCR reactions positively amplified 1.5Kbp amplicons. Sequence similarity of our 16S rRNA with that of other DNA sequences in GenBank has revealed 99% similarity to *B. oleronius* (AY988598.1) 16S rRNA. The 16S rRNA sequence was deposited in GenBank nucleotide database under the accession number GQ288406. Phylogenetic tree of our isolate represented the formation of specific clade with other *Bacillus* species. Figure 1 represents the 16S rDNA phylogenetic neighbor joining tree, showing the relationship between our *B. oleronius* strain SJC04 and known genera forming distinct clusters.

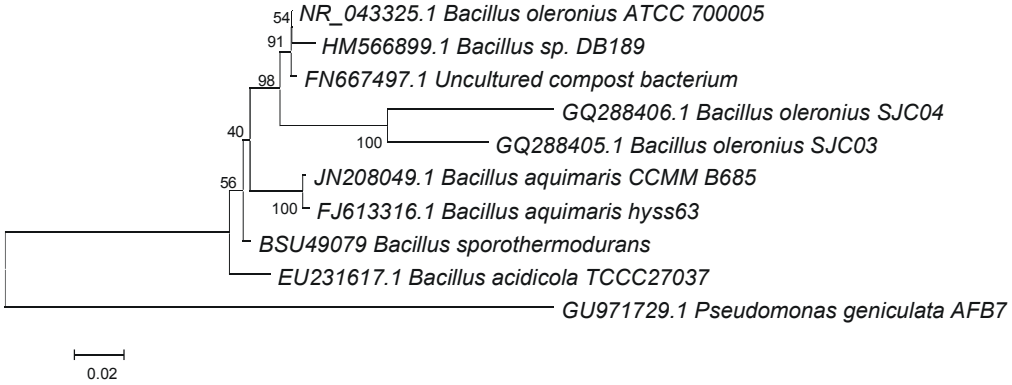

Fig. 1: Phylogenetic tree of *B. oleronius* SJC04 with its evolutionary relatives

Table 1: Bacterial cells viability in the presence of organic solvents

			Cell viability in the presence of organic solvents (CFU.ml^{-1})	
			B. oleronius	
Solvent	Log P_{OW}	Solvent conc. (v/v)	Control (Without organic solvents)	Test (Treated with organic solvents)
Cyclohexane	3.4	20%	238±80	182±34
Hexane	3.9	20%	146±80	0
Toluene	2.6	20%	212±59	31±7
Petroleum ether	NA	20%	223±46	29±6

NA - Not available

Table 2: Preliminary efflux pump assay

Sample	Growth
Control (Untreated)	Confluent
CCCP	No Growth
DNP	Confluent
Cyclohexane	No Growth
Hexane	No Growth
Xylene	No Growth
Toluene	No Growth
Petroleum ether	Confluent
CCCP+Cyclohexane	No Growth
CCCP+Hexane	Single Colony
CCCP+Xylene	No Growth
CCCP+Toluene	Single Colony
CCCP+Petroleum ether	Confluent
DNP+Cyclohexane	No Growth
DNP+Hexane	No Growth
DNP+Xylene	No Growth
DNP+Toluene	No Growth
DNP+Petroleum ether	Confluent

Organic Solvent Tolerance of *B. Oleronius*

Bacterial Viability in Organic Solvents: The tolerant nature of *B. oleronius* was studied by incubating the bacterial isolate with 20% v/v organic solvents in LB Broth (Table 1). The results revealed that toluene and petroleum ether are toxic for *B. oleronius* as the viable colonies were reduced much in treated cells than untreated control. However cyclohexane was less toxic for bacterial cells than other solvents. Hexane treated bacterial isolate has failed to produce viable colonies indicating the toxic nature of hexane in killing bacterial cells than other organic solvents tested.

Efflux Pump Assay

Rhodamine B Accumulation: The existence of solvent efflux pumps was analyzed by rhodamine B accumulation (100 µg•ml^{-1}) in bacterial cells treated with test solvents by exploring the fluorescence of Rhodamine B in bacteria under UV light. There was an accumulation of Rhodamine B dye in organic solvents treated bacteria compared to control when examined under UV light.

Efflux Pumps Inhibitors and Organic Solvent Tolerance: The effect of protonophores CCCP and 2,4 DNP (80µg/ml) on the solvent tolerance of *B. oleronius* in the presence and absence of organic solvents was studied (Table 2). No growth was observed in *B. oleronius* treated with CCCP, Cyclohexane, Hexane, xylene, toluene, CCCP + Cyclohexane, CCCP + Xylene, DNP + Cyclohexane, DNP + Hexane, DNP + Xylene and DNP + Toluene, but confluent growth was observed in *B. oleronius* treated with DNP, Petroleum ether, CCCP + Petroleum ether and DNP + Petroleum ether. Single colony growth was observed in CCCP + Hexane and CCCP + Toluene.

Antibiotics, Heavy Metals and Detergents Potentiation of Solvent Tolerance: There is a strong linkage existing between the antimicrobials resistance and solvent tolerance in *B. oleronius* as evident by comparing the abilities of the isolate to survive in an overlay consisting of 100% solvent with and without prior exposure to antibiotics, heavy metals and detergents (Table 3). On LB agar control medium (treated only with solvents), *B. oleronius* has shown confluent and single colony growth for both hexane and petroleum ether respectively. No growth was observed in control LB and test agar plates treated with cyclohexane, xylene, toluene and their combination with antimicrobials. Heavy metals potentiation was seen as confluent growth of *B. oleronius* treated with potassium dichromate + hexane. Single colony growth in was observed in SDS + hexane treatment. However an interesting result was observed in petroleum ether + potassium dichromate treated *B. oleronius*, in which the single colony was transformed to confluent growth which revealed that the cell survival

Table 3: Tolerance of bacteria to organic solvent overlays with and without prior treatment with antimicrobials

		Control	Antibiotics		Heavy Metals		Detergents	
Bacteria	Organic solvents	(Treated only with solvents)	Geneticin (500ng/ml) + Organic solvents	Hygromycin (10µg/ml) + Organic solvents	Potassium dichromate (20µg/ml) + Organic solvents	Cadmium chloride (20µg/ml) + Organic solvents	SDS (10µg/ml) + Organic solvents	CTAB (5µg/ml) + Organic solvents
B. oleronius	Cyclohexane	NG	NG	NG	NG	NG	NG	NG
	Hexane	C	NG	NG	C	NG	S	NG
	Xylene	NG	NG	NG	NG	NG	NG	NG
	Toluene	NG	NG	NG	NG	NG	NG	NG
	Petroleum ether	S	NG	NG	C	NG	NG	NG

C – Confluent growth S – Single Colony NG – No Growth

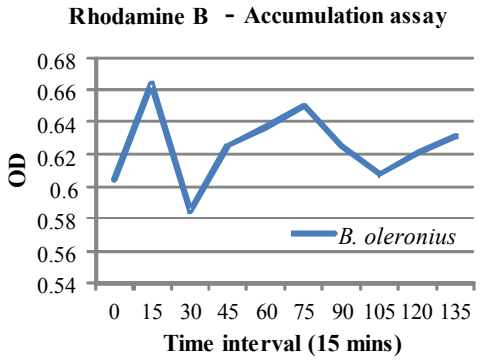

Fig. 2: Rhodamine B accumulation assay

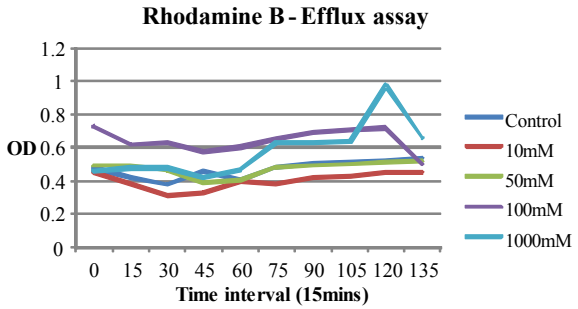

Fig. 3: Rhodamine B efflux assay with varying concentrations of glucose

was dramatically enhanced by prior incubation with 20 µg of potassium dichromate per ml. In general, heavy metals were found to transform the single colony to confluent growth and survival of cells by prior incubation with potassium dichromate.

Rhodamine B Accumulation Assay: The accumulation assay was performed to determine the activity of efflux pumps in the bacterial cells. Rhodamine B was utilized as a test dye for the accumulation assay. *B. oleronius* started accumulating dye at 15th min, after which there was a sudden decline in the accrual of dye till 30 min followed by gradual rise till 75 min and further gradual decrease in the accumulation of dye (Figure 2).

Rhodamine B Efflux Assay: B. oleronius has shown high efflux activity of rhodamine B at 1000mM (glucose containing PBS) followed by 100, 50 and 10mM compared to control (glucose free PBS). *B. oleronius* incubated with glucose has shown high efflux activity compared to untreated. Efflux activity was observed at 45 min after the incubation (Figure 3).

DISCUSSION

The aim of this work was to analyze the role of efflux pumps in providing the solvent tolerance of bacteria. In stressful environmental conditions, bacteria are constantly under threat from stressors and have developed protective mechanisms of siphon out wide range of antibiotics, detergents, dyes, organic solvents and heavy metals out of the cells by efflux pumps [23, 38]. Gram negative bacteria are the potent competitors and survivors than Gram positive bacteria in tackling the organic solvents due to the over expression of efflux pumps. An effort was taken to find out the role of efflux pumps in exhibiting the tolerance of *B. oleronius* against organic solvents.

Comparative analysis of our 16S rRNA sequence with other deposited sequences in GenBank database revealed that most of them had sequence similarities of 98% to 99% to sequences of *Bacillus* genera. Phylogenetic tree has revealed that our isolate has closely related to *B. oleronius* strain SJC03 isolated from petroleum contaminated soil and deviates evolutionarily much from other *Bacillus* species. Neighbor joining tree has revealed the specific clade formation of our isolate with *Bacillus sporothermodurans*, *B. aquimaris* and *B. acidicola* that extensively sporulates under harsh environmental conditions. *B. aquimaris* has been reported to exhibit organic solvent tolerance [24].

Efflux pumps are the proteins which extrude antibiotics, drugs and solvents out of cells. Bacteria have the ability to withstand solvent stress to certain extent as

the excess concentration will kill the bacteria by damaging its cell membrane. Our results of bacterial viability in solvents was consistent with the reports published earlier stated the toxic nature of toluene and petroleum ether in killing the bacteria [25-27]. These results have shown that organic solvents with low Log P_{ow} (1-3.5) have lethal effects in the survival of microorganisms [28, 29]. The loss of viability of bacterial isolates by toluene, hexane and petroleum ether is due to disorganization of the cell membrane and abundant binding to viable cells thus killing the bacteria [9, 20].

Rhodamine dyes are the preferred substrates for efflux pumps [30]. Examination of *B. oleronius* colonies treated with rhodamine B dyes in the presence organic solvents under UV rays has shown the accumulation of dye compared to control. This is consistent with the report stating the accumulation of Rhodamine 6G by efflux pumps in bacterial cells treated with organic solvents [20].

EPI are the chemicals which inactivate the efflux pumps of prokaryotic and eukaryotic cells. Inactivation of efflux pumps by EPI will results in the loss of efflux activity thus results in the accumulation of toxic solvents within cell which ultimately lead to the death of bacterial cells. Our results have conclusively proved that the *B. oleronius* treated with EPI and EPI + organic solvents has results in the loss of cell viability by the inactivation of efflux pumps, thus killing the bacteria. This is consistent with the reports stating that 2,4 DNP and CCCP are potent inhibitors of RND class of efflux pumps [31, 32]. Complete inhibition of bacterial growth was seen in colonies pretreated with CCCP and confluent growth in DNP pretreated bacteria. We speculate that the protonophores CCCP could be a good EPI agent for efflux pump activity studies than DNP which is a less potent EPI. In general, pre-treatment of bacterial isolates with CCCP and DNP followed by treatment with test solvents has shown that the knocking out of efflux pumps will results in the reduction or loss of organic solvent tolerance in bacterial isolates [10, 33]. This has clearly indicated the close interrelation between efflux pumps and organic solvent tolerance of bacteria. This protocol could be a simple and preliminary assay to analyze the efflux pump activity in the bacterial cells.

The presence of solvents in the contaminated site may lead to the emergence of solvent tolerant strains and therefore, solvent tolerance propagates antimicrobials (antibiotics, heavy metals, dyes and detergents) resistant bacteria [34, 37]. However, the reversal is also possible in which the antimicrobials potentiation or treatment may lead to the emergence of enhanced solvent tolerant bacteria. In both cases, the ultimate results will be the simultaneous activation of solvent tolerance and antimicrobial resistance which will make the bacteria to become "superbugs" in the causal of diseases. Our results are consistent with the report which stated the transformation of bacterial single colony to confluent growth with the prior treatment of tetracycline and exposure to organic solvents [35]. The report also stated that the pretreatment of bacteria with tetracycline antibiotics has greatly increased the cell survival by activating the efflux pumps thereby enhancing the solvent tolerance of isolates. This is the first report to state that the heavy metals potentiation can enhance the solvent tolerance in acne rosacea causing *B. oleronius*. This also alarmed the suspicion that *B. oleronius* subjected to heavy metals and organic solvents in petroleum contaminated site could make use of efflux pumps to protect them from hydrocarbons rich environment and confer them a competitive advantage in the infection of humans.

Accumulation of Rhodamine B was observed in *B. oleronius* cells. Effluxing of rhodamine B was more in glucose treated cells than control. This has shown that the glucose has provided energy necessary to efflux rhodamine B in and out of the cells. This is consistent with the report stating that the siphon out of rhodamine 6G dye in *Saccharomyces cerevisiae* in the presence of energy providing glucose for the efflux pumps [36]. This has clearly indicated that the dye could act as a substrate for driving efflux pump activity.

Due to the dual presence of organic solvents and heavy metals in petroleum contaminated site, it is therefore likely to conclude that the tolerance and resistance mechanisms of bacteria are interrelated and activation of one will activate the whole set of resistances in dwelling microbes thereby increasing their chances of acquiring multi-antimicrobials extrusion and disease causing abilities.

The present study involved the characterization of *B. oleronius* in analyzing the role of efflux pumps in organic solvent tolerance. Cell viability has greatly decreased in solvent treated cells compared to control. The protonophores CCCP and 2,4 DNP coupled with the treatment of organic solvents has resulted in the dramatic reduction of cell survival and growth indicating that the efflux pumps are essential for tackling the solvent stress. Antimicrobial potentiation of solvent tolerance has revealed that the antimicrobials can activate the efflux system which in turn enhances the solvent tolerance of bacteria. Rhodamine B accumulation

and efflux activity in bacterial isolates divulged the critical importance of efflux pump in the extrusion of harmful compounds for the survival of bacteria under stressful environment. The solvent tolerant bacteria could be an ideal candidate for genetic studies of stress response of bacteria and in the bioremediation of petroleum contaminated site.

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