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Isolation and Characterization of Non-Adapted BTEX Degrading Bacterial Strains from Petroleum Contaminated Environment

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The volatile monoaromatic hydrocarbons BTEX have been termed and considered as environmental pollutants. In this study, the significance of bacterial isolates in biodegradation of Benzene, Toluene, Ethyl benzene and o-Xylene (individually) were analysed. Seven different isolates were isolated from two different sources namely petroleum effluent and petroleum contaminated soil. After initial characterization of these isolates the growth pattern of them with BTEX as sole carbon source was carried out and the degradation potential were studied. The isolate *Bacillus pumilus* MVSV3 was identified and found to be effective degrader of BTEX. When 50 mg/L of BTEX was supplemented individually, *Bacillus pumilus* MVSV3 showed 56% of benzene, 59% of toluene, 55% of ethylbenzene and 54% of o-Xylene degradation. The bacterial isolate had potential to tolerate different metal ion concentration (0.5-0.25 mM) and varying percentage of salinity (2-10 %). The GCMS studies revealed that it could degrade BTEX into catechol and muconic acid, which are the major intermetabolites formed during an ortho pathway mediated degradation of monoaromatic hydrocarbons. These observations indicated that this strain has a good potential for degrading BTEX and could contribute significantly to biodegradation of monoaromatic compounds.

Key words: BTEX; *Bacillus pumilus*; Logistic growth; Scanning Electron Microscope; HPLC, GCMS.

Aromatic compounds are the second most abundant family of organic constituents present in nature after carbohydrates. Benzene, Toluene, Ethyl benzene and Xylenes; also known as BTEX are volatile monoaromatic hydrocarbons commonly present in crude petroleum and petroleum products such as gasoline[1]. They are also produced as bulk chemicals for industrial use as solvents and starting materials for the manufacture of pesticides, plastics and synthetic fibers[2].One of the most common ground water and soil contaminants are the BTEX, which account as much as 90% of the gasoline components. They possess major threats due to their high toxicity and solubility in water relative to other petroleum hydrocarbons[3, 4]. The US Environmental Protection Agency (US EPA) classifies them as Environmental priority pollutants since they are highly toxic and carcinogenic to humans[5]. Traditional degradation techniques involving absorption, adsorption, combustion and condensation reflects several drawbacks as high operating cost, cost of maintenance, need of high energy input and production of other toxic byproducts. Biodegradation is an ecofriendly method that depends on natural ability of microorganism to degrade toxic pollutant into less harmful products such as carbon dioxide and water[6]. Thus the removal of BTEX from the environment by cost efficient biodegradation method has become a major field of interest[7]. The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic

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conditions.Accidental spills and industrial discharges have resulted in pollution of the environment with BTEX[8]. Furthermore, gasoline leakage from underground storage tanks has been identified as an important source of groundwater contamination with BTEX. The mobility and toxicity of the BTEX compounds are of major concern compared with other oil hydrocarbons, BTEX are relatively water-soluble and therefore contamination in the groundwater level is very easy and rapid[9]. The EPA (U.S.) has established permissible levels for these contaminants in drinking water in terms of maximum contaminant level (MCLs). The MCLs for BTEX are 0.005, 1, 0.7 and 10 mg/L respectively. The present study is aimed to isolate and study the degradation potential of bacterial strains from petroleum effluent and petroleum contaminated soil. Here the source for pollutant degradation is provided by the pollutant themselves; and therefore investment and cost of operation are lowered. After initial characterization of these isolates the growth pattern of them with BTEX as sole carbon source has been carried out and the degradation ability were analyzed.

MATERIAL AND METHODS

Bacterial strains and cultivation conditions

Petroleum effluent was collected from a petroleum refinery, Chennai, Tamil Nadu as a source of isolation[10]. A second source, the contaminated soil sample was collected from the top 50 cm of the disposal site in Ennore, Tamil Nadu and soil samples were then passed through a 2 mm sieve. All samples were placed into sterile bottles and polyethene bags and stored at 4°C[11]. Isolation procedures were carried out using spread plate method and isolates that grew as single pure colony in Luria Bertani (LB) media with BTEX as a sole carbon source were selected for further studies. The isolates are cultured in LB media slants and plates and preserved at 4 °C. These strains were sub cultured with 50 mg/L of BTEX in 100 mL of mineral salt medium (MSM) containing (g/L): 1.0 g KH_2PO_4, 1.25 g Na_2HPO_4. 2 H_2O, 0.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$. 7 H_2O, 0.5 g $CaCl_2$. 2 H_2O, 0.005 g $FeSO_4$. 7 H_2O and then incubated in 250 ml flask at 37°C on rotary shaking incubator at 150 rpm. All the experimental contents were sterilized for 20 min at 121°C and 15 lbs in an autoclave before inoculation[12].

Characterization of BTEX degrading strains

The seven isolated bacterial strains were subjected to morphological observation and analyzed for phenotypic characters using "Enterobacteriaceae identification kit", (Himedia, India). The basic study of Gram staining was also carried out. Other tests included the glucuronidase, nitrate reduction, lysine decarboxylase, orinithine decarboxylase, phenylalanine deamination, arabinose, oxidase and citrate utilization tests. The bacterial genomic DNA was isolated by alkali lysis method[13]. Bacterial 16S rDNA was amplified from the extracted genomic DNA by using the universal eubacterial 16S rDNA primers, fD1 - 5'-GAG TTT GAT CCT GGC TCA-3' and rP2 - 5'-ACG GCT AAC TTG TTA CGA CT-3'. Conditions for PCR used were, 94°C initial denaturation for 2 min, 1 min of denaturation at 95 C in 35 cycles, one min extension at 72°C and 10 min of final extension at 72°C. The cyclic sequencing reaction was performed using BigDye terminator V3.1 cycle sequencing kit containing Ampli Tac DNA polymerase. The reaction was then purified on sephadex plate by centrifugation to remove unbound labelled and unlabeled nucleotides and salts. The purified reaction was loaded on to the 96 capillary ABI 3700 automated DNA analyzer and electrophoresis was carried out for 4 h. The nucleotide sequences are registered in the computer attached with the ABI 3700 DNA analyzer[14]. The nucleotide sequences obtained from the ABI DNA analyzer were identified using BLAST (Basic Local Alignment Search Tool) software available in NCBI (National Centre for Biotechnology Information). The sequence was then analyzed with BLAST software to identify the specific type of bacteria corresponding to the nucleotide sequence. Phylogenetic tree was constructed by neighbor-joining analysis method to compare the other available 16S rRNA sequences using an automatic alignment tool (Clustal W)[15].

Scanning electron Microscopy (SEM)

SEM analysis of the efficient organism was carried out to study its morphological structure. Samples for SEM were prepared by growing *Bacillus pumilus* MVSV3 on MSM with BTEX for 48 h. The culture was centrifuged for 10000 rpm for 10 min and the pellets were suspended in 2% glutaraldehyde with 0.05

phosphate buffer and 4% sucrose[16].The sample was air dried by placing it on aluminum foil. The dried sample was then placed on carbon coated SEM grids and sputtering was done with EMITECH SC 7620 for gold coating. SEM studies were done using TESAN VEGA3 SBU (Czech Republic) at 11 kV of volts.

BTEX degradation studies

Each isolates were inoculated to four different conical flasks with 100 mL of MSM containing 50mg/L of Benzene, Toluene, Ethyl benzene and o-Xylene (BTEX) added individually. The pH was maintained at 7. The cul-ture medium was placed on a shaker (150 rpm) at 37º C. Growth was monitored by measuring the dry biomass obtained for every 24 h. BTEX degradation was analyzed using HPLC after 5 days of incubation. Samples were centrifuged (10 min, 10000 rpm) (Remi R 24, India) to separate cell mass and the supernatant. The samples were extracted in organic solvent (n-hexane) for analysis. The extracted samples were injected in a high-performance liquid chromatography system (HPLC,Shimadzu, Japan) equipped with a UV-vis Detector and C – 18 column. The samples were analyzed using the following programme: mobile phase Acetonitrile-water 75: 25, wavelength 254 nm, flow rate 1 mL/min[17, 18, 19]. Degradation efficiency is calculated using the formula:

$$\text{Degradation efficiency (\%)} = \frac{(C_i - C_f)}{C_i} \times 100\ \% \quad ...(1)$$

Where, C_i - initial concentration of substrate, C_f - final concentration of substrate

Growth Kinetics

When a limited concentration of substrate is provided to the bacterial isolates logistic growth pattern is observed. This gives us the maximum biomass of the isolates that can survive in that condition. The logistic growth modelling of maximum BTEX degrading isolate was carried out and fitted with the following equation[20];

$$X = \frac{X_0 . e^{kt}}{1 - \frac{X_0}{X_c}(1 - e^{kt})} \quad ...(2)$$

Where X_0 denotes the initial biomass (0.05g/L), k is the carrying capacity coefficient (h^{-1}), X_c is the carrying capacity (g/L) and t is the incubation time (h).

GCMS analysis

Gas chromatography and mass spectrometry analysis of the best degrading bacterial isolate *Bacillus pumilus* MVSV3 on mixed BTEX was carried out to identify the intermediates formed during degradation. The degradation ability of MVSV3 on BTEX was determined by growing it in 250mL Erlenmeyer flask containing 100 mL of MSM supplemented with 50 mg/L of BTEX (mixed). After five days of incubation at 150 rpm, 30 °C and pH 7 the cell suspension were clarified by centrifugation at 10000 rpm for 10 min. The supernatant was later extracted with equal volume of hexane and filtered using 0.2 µm Agilent filter syringe before analysis. The GCMS analysis was carried out by JEOL GC MATE II (USA), with front inlet temperature of 220° C. The flow rate was preset to 1 mL/min. The oven temperature was varied from 50 to 250 at 10° C /min, equipped with mass analyzer: quadruple double focusing mass analyzer, detector: Photon multiplier tube. All metabolites were identified by GCMS by matching retention time and ion spectra with authentic standard and NIST library data[21].

Tolerance of MVSV3 on metal ions and salinity:

Sometimes the point of hydrocarbon contamination may also harbor metal ions and salinity in significant value, and these factors may also contribute for the degradation of the contaminants. Some metal ions may also enhance or degrade the enzyme activity of the bacteria. Hence tolerance of *Bacillus pumilus* MVSV3 towards metal ions (Zn^{2+}, Pb^{2+}, Ni^{2+} and Cd^{2+}) and salinity was carried out by determing the growth biomass of the isolate. Specified amount of selected metal ions (0.5-0.25 mM) and NaCl (2-10%) was added to the growth medium MSM adjusted to pH 7[22,23]. As a sole carbon source BTEX was provided as a mixture at 50 mg/L concentration each. Growth on other compounds were also monitored.

RESULTS AND DISCUSSION

Bacterial isolation and Identification

In this study, two different sources were selected for isolation. Petroleum effluent as well as the soil contaminated with petroleum were chosen

so that potential strains were isolated. Two strains MVSV1 and MVSV2 were isolated from the petroleum effluent and five strains MVSV3-7 were isolated from petroleum contaminated soil. The morphological, physiological, biochemical characteristics (Table 1) and the comparative analysis of DNA sequence with available database showed that the seven isolates were *Ochrobactrum intermedium* MVSV1, *Ochrobactrum spp* MVSV2, *Bacillus pumilus* MVSV3 , *Bacillus cereus* MVSV4, *Alcaligenes faecalis* MVSV5 , *Ochrobactrum spp* MVSV6 and *Alcaligenes faecalis* MVSV7. These isolates have been submitted in NCBI under the following accession numbers, JN089705, JN089706, JN089707, JN089708, JN089709, JN089710, and JN089711. Percentage similarity values were obtained after pairwise alignment of the sequences of 16S rDNA of the strains and EMBL database sequences, and the sequences giving the highest scores were retrieved to construct the phylogenetic tree (Fig. 1A).

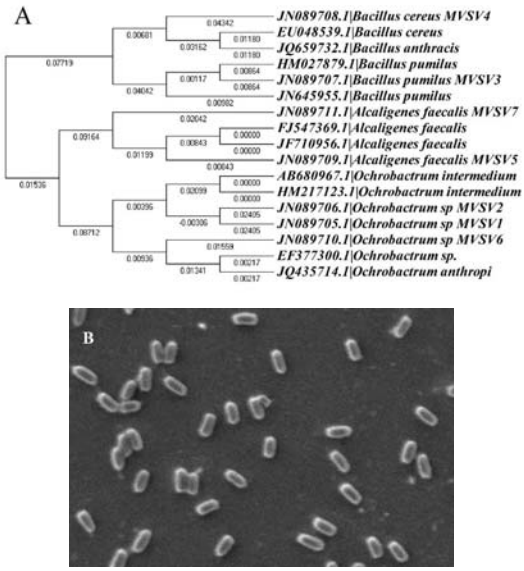

Fig. 1. (A) Phylogenetic tree of seven bacterial isolates, (B) *Bacillus pumilus* MVSV3 under Scanning electron microscope

Bacterial isolate MVSV3 and MVSV4 were found to be gram positive that could utilize glucose and sucrose but were found to be negative for lactose and sorbitol. Whereas bacterial isolates MVSV1, 2, 5, 6 and 7 were identified as Gram negative stains that could utilize both lactose and glucose. Fig. 1B represents the SEM image of *Bacillus pumilus* MVSV3.

BTEX degradation by isolates

Petroleum contaminated soil was found to be the most promising source to isolate BTEX degrading bacteria. Degradation potential of all 7 isolates were examined for all the four monoaromatic compounds. All the isolates could grow well on media supplied with BTEX a sole carbon source (Fig. 2A, 2C, 3A and 3C). Analysis revealed that, degradation of all the 4 compounds (50 mg/L of BTEX) was efficiently done by *Bacillus pumilus*

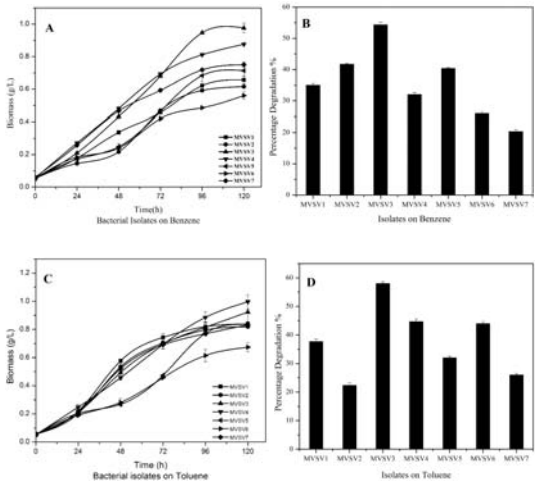

Fig. 2. Bacterial isolates growth (A, C) and degradation (B, D) on Benzene and Toluene, pH 7, 37 °C, substrate concentration 50mg/L each and 1% inoculum

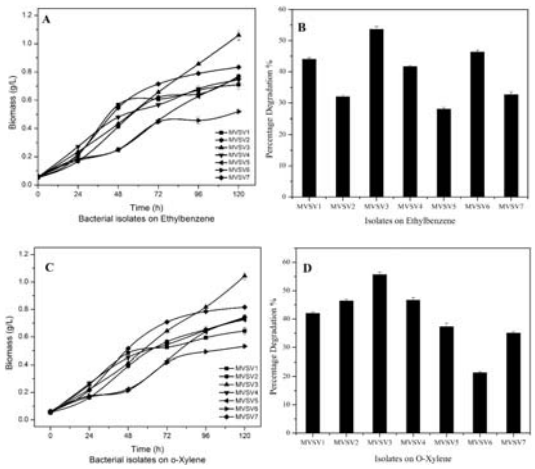

Fig. 3. Bacterial isolates growth (A, C) and degradation (B, D) on Ethylbenzene and o- Xylene, pH 7, 37 °C, substrate concentration 50mg/L each and 1% inoculum

MVSV3 (Fig. 2B, 2D, 3B and 3D).

It could degrade benzene (56%), toluene (59%) ethylbenzene (55%) and o-xylene (54%) when compared *to Alcaligenes faecalis* MVSV7 that degraded BTEX only upto 21, 27, 33 and 36% respectively. Similar results were found by Babaarslan *et al*.,[24], non-adapted *Psuedomonas stutzeri* and *vibrio mimicus* degraded 20 mg/L of benzene, ethylbenzene and o-xylene up to 55,100,100% in 31 days where as it could degrade 100% of toluene in 4 days. Many studies over degradation of BTEX and other polyaromatic hydrocarbons had been carried out with other potential organisms such as *Pseudomonas spp*[25], *Rhodococcus spp*[26,27], *Mycobacterium*[19] and many fungal strains such as *Cladophialophora sp*[28]. Therefore the findings made in this study shall be useful in investigating the novel *Bacillus pumilus MVSV3* organism in field of BTEX degradation.

This strain was also further tested for utilization of other substrates to elucidate more on its array of degradation ability and was found to grow on polyaromatic compound like phenanthrene, which shall be a better potential for biodegradation (Table 2).

Growth model

Survival of bacterial isolates in BTEX supplemented medium is a key deciding factor in the degradation of compounds present in liquid phase[29]. The growth biomass obtained for *Bacillus pumilus* MVSV3 was fitted with the logistic equation (Fig. 4), and the kinetic parameters carrying capacity (X_C), carrying capacity coefficient (k) were tabulated in Table 3. At 50mg/L of BTEX

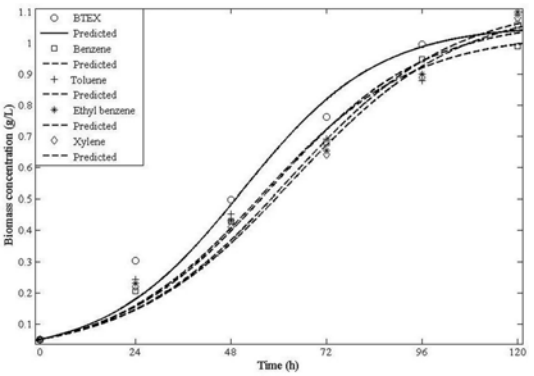

Fig. 4. Logistic equation used to fit growth pattern of *Bacillus pumilus* MVSV3 isolate on BTEX

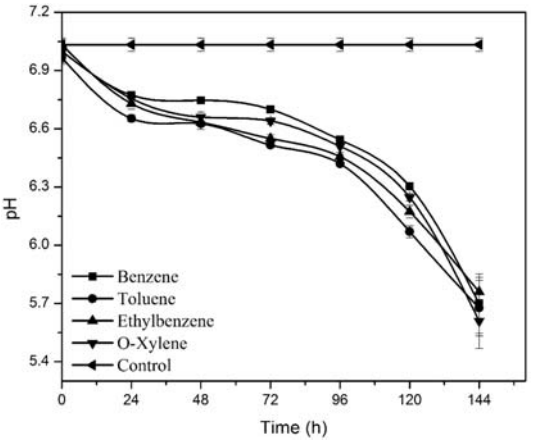

Fig. 5. Change in pH during degradation of BTEX by *Bacillus pumilus* MVSV3

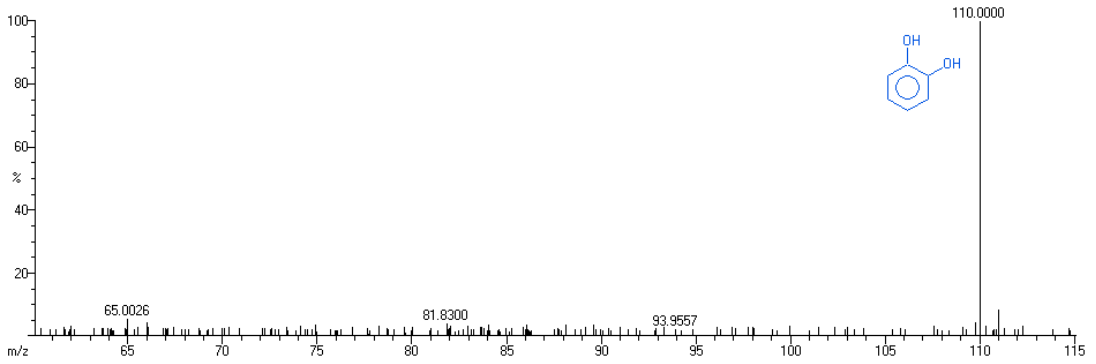

Fig. 6. Mass chromatogram of catechol (m/z= 110) formed during BTEX degradation by *Bacillus pumilus* MVSV3

concentration the dry biomass of the isolate increased in density during its log phase, further showed a small stationary phase and finally attained the decline phase after 120 h of incubation.

The maximum carrying capacity was found to be in the range of 1.029 to 1.125 g/L and the maximum carrying coefficient k was found to be 0.048- 0.058 h^{-1} .

The R^2 value obtained were above 0.97, which showed that growth of the isolate fits well into the logistic model. Degradation of BTEX (mixed) by *Bacillus pumilus* MVSV3 and its growth on the mixture showed a decrease in pH during incubation (Fig. 5). It attained an acidic pH of 6.2- 5.9 from initial maintained pH 7 indicating the formation of acidic intermediates during degradation of BTEX. This finding was similar to studies carried out in biodegradation of phenanthrene using adapted microbial consortium, a total of three bacterial monocultures *Sphingobacterium sp.*, *Bacillus cereus* and a novel bacterium *Achromobacter insolitus* MHF ENV IV[30].

Tolerance of MVSV3 on metal ions and salinity

MVSV3 isolate could successfully grow on a culture medium supplemented with metal ions and NaCl (5 days of incubation). Higher concentration of Cd^{2+} proved to be critically toxic for the bacteria. The maximum growth was achieved with Zn^{2+} ions at 0.1mM. Pb showed lesser changes in the biomass and Ni ions showed decrease in biomass concentration as Ni metal ion concentration increased (Table 4a). Thus it proves that the toxicity increased in the order as $Zn^{2+} < Pb^{2+} < Ni^{2+} < Cd^{2+}$ which is similar to the findings of Volker *et al.*, [22] who studied the degradation of diesel fuel by a microbial community from a soil polluted with heavy metals. The addition of NaCl to the culture medium also proved that the organism could withstand up to 6% NaCl (Table 4b) and hence predicting that the isolate MVSV3 is slightly halophilic in nature.

GCMS

During the biodegradation of BTEX by MVSV3 isolate, there was a change in the

Table 1. Biochemical characterization of isolates that degrade BTEX

S.N	Tests	MVSV1	MVSV2	MVSV3	MVSV4	MVSV5	MVSV6	MVSV7
1.	Grams Staining	–	–	+	+	–	–	–
2.	Methyl Red	+	+	–	–	–	+	–
3.	Voges Proskauer	–	–	+	+	–	–	–
4.	Citrate	+	–	+	+	+	+	+
5.	Indole	+	+	+	+	–	+	–
6.	Glucuronidase	+	+	–	–	–	+	–
7.	Nitrate reduction	–	–	+	+	–	–	–
8.	Lysine decarboxylase	+	+	+	+	+	+	+
9.	Lactose	+	+	–	–	+	+	+
10.	Glucose	+	+	+	+	+	+	+
11.	Sorbital	+	+	–	–	–	+	–
12.	Sucrose	+	+	+	+	–	+	–
13.	Orinithine decarboxylase	+	+	+	+	+	+	+
14.	Urease production	–	–	–	–	+	–	+
15.	PhenylalanineDeamination	–	–	–	–	–	–	–
16.	H2S production	–	–	–	–	–	–	–
17.	Arabinose	+	+	–	–	–	+	–
18.	Oxidase	+	+	–	–	–	+	+

Table 3. Growth kinetic parameters obtained for *Bacillus pumilus* MVSV3 isolate using logistic equation

Substrate (mg/L)	Carrying capacity X_c (g/L)	Carrying capacity coefficient k (h^{-1})
BTEX	1.057	0.058
B	1.029	0.053
T	1.074	0.051
E	1.125	0.048
X	1.117	0.048

transparent medium to a turbid medium indicating the growth of isolate as well as accumulation of intermediates formed. GCMS analysis and comparison with the standard m/z library identified the presence of the major intermediates (Fig. 6) catechol (m/z 110) and muconic acid (m/z 142) indicating the ortho ring cleavage pathway of BTEX. This finding is similar to Otenio *et al.,* [5] and Haibo *et al.,* [31] had also reported the presence of catechol and muconic acid as major intermetabolites produced during BTX degradation. Georg *et al*.,[1] stated that aerobic degradation of aromatic compounds produce catechol as central intermediates and then the produced catechol acts as substrates for ring cleaving dioxygenases in the lower pathway. Other metabolites detected in the aerobic degradation of BTEX were acetic acid, benzyl alcohol, acetophenone and propanol[32]. These finding suggests the higher efficiency of *Bacillus pumilus* degradative enzyme system that might have surpassed the inhibitory effects of hydrocarbons.

BTEX are toxic environmental pollutants that commonly occur as groundwater contamination with the release of petroleum products[16]. BTEX acts as both substrate for growth of the organism and as dominant pollutants in the environment Degradation of BTEX can be achieved once the resonance energy the ring structure uses to be stable is broken[1]. This can be done by both aerobic and anaerobic method of degradation.

Decrease in residual amount of BTEX and presence of catechol as determined by GCMS during biodegradation exhibited similarity with other reported bacteria such as *Janibacter sp* SB2[33]. *Pseudomonas putida* BCNN 106[34] and fungi *Paecilomyces vaviotii*[35] . Similarly authors have reported the isolation of bacterias belonging to Enterobacteriaceae family, Pseudomonas and Azotobacteriaceae which were isolated from hydrocarbon contaminated soil[36] . Though many research over bacteria of other genera is explored , very few papers have reported on *Bacillus spp* in degradation of hydrocarbons[37]. It is also postulated that *Bacillus spp* due to their resistant endospores are more tolerant to high concentration of hydrocarbons in soil[38]. Generally *Bacillus pumilus* is studied as an environmental organism which is primarily investigated for its various enzyme production and application[39].

CONCLUSION

In the present study, we had isolated a novel *Bacillus pumilus* MVSV3 bacteria that could

Table 4. (a) Tolerance of *Bacillus pumilus* MVSV3 on metal ions

Concentration (mM)	Maximum Biomass (g/L) on Zn^{2+}	Maximum Biomass (g/L) on Pb^{2+}	Maximum Biomass (g/L) on Ni^{2+}	Maximum Biomass (g/L) on Cd^{2+}
0	1.1	1.08	0.99	1.08
0.05	1.15	1.05	0.84	0.78
0.1	1.42	1.07	0.76	0.64
0.15	0.76	1.07	0.56	0.41
0.2	0.37	1.01	0.34	0.34
0.25	0.32	1	0.13	0.10

(b) Tolerance of *Bacillus pumilus* MVSV3 on NaCl

NaCl (%)	Maximum Biomass (g/L)
0	1.09
2	1.13
4	1.34
6	1.64
8	0.089
10	0.042

degrade monoaromatic hydrocarbons such as benzene, toluene, ethylbenzene and o-xylene to a better extend even without being acclimatized to it. Decrease in growth and degradations during increase in substrate concentration reflects the lack of acclimatization of the newly isolated strain to BTEX. Further adaptaion of *Bacillus pumilus* MVSV3 to higher concentration and optimization of other growth parameters can be a breakthrough in enchancing the degradation ability of the organism.It also suggested that the bacterial strain has not only been highly potential in degrading the monoaromatics but could also utilize other toxic substrates.This study hence adds a guideline in elucidating the ability of BTEX degrading strains of *Bacillus spp* from petroleum contaminated environment.

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