International Journal of Science and Research (IJSR)
ISSN: 2319-7064
Impact Factor 2024: 7.101

Biodegradation of Benzene and Toluene Compounds by Highly Effective Phyllosphere Bacteria: An Assertive Strategy for Environmental Remediation

John K Mathew[1], Tamilselvan N.[2]

[1]Microbiology, Adhiparasakthi College of Arts and Science (Affliated to Thiruvalluvar University), GB Nagar,Kalavai, Ranipet district, Tamilnadu 632506, India
Email: *madhunimathew[at]gmail.com*

[2]Microbiology, Adhiparasakthi College of Arts and Science (Affliated to Thiruvalluvar University), GB Nagar,Kalavai, Ranipet District, Tamilnadu 632506, India.
Email: *tamil.tamiln[at]gmail.com*

Abstract: ***The key petroleum aromatic hydrocarbons—benzene, toluene, ethylbenzene, m-xylene, o-xylene, and p-xylene—are known as BTEX compounds and contribute significantly to environmental pollution. Research indicates that phyllosphere bacteria can enhance the degradation of these harmful compounds, with Pseudomonas putida HLPSC8 showing the highest hydrocarbon degradation potential among two tested strains. A tolerance study demonstrated this bacterium's effectiveness at varying concentrations of BTEX compounds (1% to 10%), measured using the gravimetric method. Optimizing biodegradation activity involves factors like pH, temperature, agitation, and nitrogen sources. In growth kinetic studies, Pseudomonas putida degraded 91% of benzene and 92% of toluene after 168 hours, resulting in 15 and 12 identified byproducts, respectively. These findings will guide future research on hydrocarbons and support the biodegradation of these hazardous compounds.***

Keywords: Benzene, Toluene, Phyllosphere, Degradation, Gas Chromatography

1. Introduction

Hydrocarbons are compounds made entirely of carbon and hydrogen, existing as gases, liquids, low-melting-point solids, or polymers. They are categorized into saturated hydrocarbons, which have only single bonds, and unsaturated hydrocarbons, which contain double or triple bonds.

In the petroleum refining and petrochemical industries, six key aromatic hydrocarbons known as BTEX compounds are benzene, toluene, ethylbenzene, o-xylene, m-xylene, and p-xylene. These are typical volatile monoaromatic hydrocarbons as described by (Rahul et al.2013, Yadav and Reddy 1993). Due to the increase in petrochemical industries globally, these hydrocarbons are released into the atmosphere and can spread over large areas by wind (Durmusoglu et al.2010). The World Health Organization has identified BTEX (monoaromatic hydrocarbon compounds) as potent carcinogens. These BTEX compounds are found mainly in industrial diesel oil products and can easily volatize in the atmosphere. Indoor air may originate from outdoors, providing a baseline for indoor BTEX concentrations influenced by climatic conditions and air exchange through ventilation systems (Zhang et al.2007). Due to their armful effects, BTEX compounds are considered as top pollutants by the United States Environmental Protection Agency (Dean et al.1985). A prolonged exposure to these compounds, whether directly or indirectly, can lead to multiple disorders including genetic disorders, excretory system failure, neurological disorders, and even cancer Irwin (1997**).**

Several effective methods exist for the removal of BTEX, including bioremediation, advanced oxidation technology, photocatalysis, sonolysis, and radiolysis. Bioremediation is primarily based on biodegradation, which involves either the complete mineralisation of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein, or the transformation of complex organic contaminants into simpler organic compounds using biological agents such as microorganisms (Preethy Chandran and Nilanjana Das (2011) . The bacteria found on leaves can break down organic pollutants from the air. This is the first direct evidence of such activity and suggests that these bacteria could help naturally reduce organic air pollution (Amarjyoti Sandhu et al. 2007). Additionally, the process can be enhanced by providing nutrients such as phosphorous, nitrogen, and carbon (Maier et al.2000). BTEX degradation can follow four activation mechanisms: hydroxylation, carboxylation, methylation, and fumarate addition. Hydroxylation is one of the mechanisms that explains the transformation of benzene into phenol, toluene into benzyl alcohol or p-cresol, and ethylbenzene into 1-phenylethanol. Carboxylation to benzoate is thought to be the primary mechanism of degradation for benzene. (Diego et al.2024).

The first step in BTEX degradation involves an oxidative process that leads to the formation of catechol. The breakdown of catechol molecule occurs in a later step. Monooxygenases such as phhN, xylA, tbmD, tol, and tmoA are involved in the initial step of oxidation (Kahng et al.2001). To assess the complex molecular structure of BTEX compounds, they need to be isolated and their degradation pathway determined (Junca et al.2004). The ability and rate

of hydrocarbon biodegradation depend on the broad-spectrum activity of the selected bacterial strains (Calvo et al.2009). Aerobic bacteria that are capable of breaking down BTEX compounds can be found in nature. Research on the metabolism and genetics of BTEX degraders suggests that Pseudomonas species are primarily involved in the degradation of these compounds (Arenghi,F.L et al.2001).

Bacteria on the surface of leaves, known as phyllosphere bacteria, also contribute to the removal of hydrocarbon compounds from the air through stomatal uptake and sorption. However, these bacteria are not evenly distributed and occur in the form of single colonies or groups Monier and Lindow (2004).

The colonies of bacteria present on the aerial parts of plants are influenced by various environmental factors such as pH, temperature, wind, radiation, and agitation (Kinkal.L et al.1997). Recently, molecular methods have been used to study the bacterial communities on maize plants and how they respond to changes in ultraviolet radiation Kadivar and Stapleton (2003).
A recent study has found that the biodegradation of BTEX compound by phyllosphere bacteria is more effective. The study used a new isolate identified as Rhodococcus sp 2JUT312 via taxonomic and 16srRNA analysis, which was found to degrade BTEX compounds more efficiently than other reported o-xylene degrading bacteria. Additionally, Pseudoxanthomonas spadix BD-a5a, which was isolated from gasoline contaminated soil, has the ability to degrade all six BTEX compounds (Eun Jin Choi et al.2013).

Different bacteria use different pathways to degrade hydrocarbons. For example, some bacteria oxidize the aromatic ring using monooxygenases and dioxygenases, which oxidize the methyl or ethyl group and the aromatic ring, respectively (Jindrova et al.2002). This leads to the formation of a new compound called pyrocatechols, which is considered the top pathway for hydrocarbon degradation. In the bottom pathway, the pyrocatechol-cleaved product undergoes the tricarboxylic acid cycle through the action of catechol dioxygenases (Farhadian et al.2008, Andreoni and Gianfreda 2007).

Pseudomonas putida AQ8 has been found to be capable of degrading all three isomers of xylene - ortho, para, and meta. However, the degradation percentage is lower compared to other BTEX compounds. According to several studies (,El Naas et al.2014.You et al.2013). The presence of methyl groups in the aromatic rings makes it harder for microorganisms to oxidize xylene isomers. This difficulty can be overcome by the involvement of monooxygenases (El Naas et al.2014).

This study investigates the isolation of phyllosphere bacteria from the leaves of Punai and Pongamia trees situated near the industrial estates of Puducherry and Cuddalore SIPCOT. The isolated bacteria were screened to identify potential hydrocarbon-degrading strains using both biomass growth and gravimetric methods. Following this screening, the five most promising strains were characterized through 16S rRNA sequencing. These selected strains were subsequently tested for their tolerance to all BTEX compounds as well as their capacity to react with combined hydrocarbons. Moreover, the study assessed various factors such as pH, temperature, agitation, and different nitrogen concentrations. The degrading activity of the strains was evaluated through growth kinetic profiles and gas chromatography analysis. Additionally, the degradation pathways of BTEX compounds by the five isolates were illustrated.

2. Materials and Methods

2.1 Sampling

Leaves coated with a hydrocarbon overlayer were collected from Punai and Pongamiya trees located in the industrial area near Cuddalore (SIPCOT) and the Puducherry industrial estate, where numerous industries operate around the clock. The selected leaves were cut into pieces using pre-sterilized scissors and forceps, then placed into a pre-sterilized 1-liter container. The sample was transported to the laboratory at a temperature of 4ºC with the help of ice packs in an airtight thermocol box, and was processed immediately to isolate hydrocarbon-utilizing phyllosphere bacteria. To maintain the highest level of hygiene, all samples were handled under strictly aseptic conditions.

2.2 Isolation of hydrocarbon utilizing bacteria

The hydrocarbon spots were carefully selected and removed in a sterile environment. One gram of the extracted sample was homogenized with pre-sterilized bacteriological saline, which served as the diluent. This homogenate was then inoculated into 100 mL of Bushnell Haas broth contained within a 250 mL conical flask, and it was supplemented with 1% crude oil to serve as the exclusive carbon source for hydrocarbon-utilizing bacteria.After seven days of incubation, the broth containing the developing bacterial population was serially diluted using bacteriological saline. The diluted mixture was subsequently spread-plated onto fresh nutrient agar to isolate various bacterial species. Morphologically distinct colonies were carefully selected and repeatedly cultured on new nutrient agar plates using the quadrant streaking technique. The purity of the cultures was confirmed by examining colony morphology and conducting microscopic analysis with the Gram staining method.

2.3 Screening of potential hydrocarbonoclastic bacteria

Pure cultured bacteria were individually tested for their ability to break down hydrocarbons. The inoculum was standardized to 108 CFU/ml using a 0.5 McFarland turbidity standard. Then, 1% of the inoculum was added to a 250 ml conical flask with 100 ml of Bushnell Haas broth and 10% crude oil, and incubated for 7 days at 37ºC.Afterward, hydrocarbonoclastic strains were evaluated based on the remaining crude oil. The oil was extracted with an equal volume of hexane, and the residual oil was measured gravimetrically **(Rahul et al. 2013).** Additionally, the total dry weight of cell biomass was determined by centrifuging the broth and drying the pellet in a hot air oven at 60ºC..

2.4 Molecular identification of potential strains

The study identified potential strains based on cell biomass and crude oil biodegradation efficiency. These strains were further characterized through molecular techniques, specifically using the 16S rRNA identification method, following a DNA isolation procedure.

Primers: Universal set of the Eubac primers (Gurtler and Stanisich, 1996)
27F - 5′- AGAG TTTG ATCM TGGC TCAG -3′
1492R - 5′- TACG GYTA CCTT GTTA CGAC TT -3′

PCR Amplification: PCR was performed on an Eppendorf thermal cycler using a 50 µl reaction mix, which included 5 µl of 10× amplification buffer, 5 µl of 1.5 mM MgCl2, 1 µl of each primer, 1 µl of dNTP, and 0.25 µl of Taq polymerase. The process involved an initial denaturation at 95°C for 1 minute, followed by 35 cycles of 94°C for 35 seconds, 55°C for 40 seconds, and 72°C for 2 minutes, concluding with a final extension at 72°C for 8 minutes. The products were analyzed using a 1.2% agarose gel from Genei.

DNA Sequencing: The PCR product was purified using the Qiagen PCR purification kit and sequenced on an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA).

Phylogenetic Tree Reconstruction: We calculated evolutionary distances using three methods: the maximum Neighbour-Joining method, UPGMA, and Maximum Composite Likelihood. MEGA 11 software was used for analysis, and tree topologies were assessed with 1,000 bootstrap replicates.

2.5 Secondary screening of most potential hydrocarbons utilizing bacterium

In this study, we screened five potential hydrocarbon-degrading bacterial strains, identified at the molecular level, for their ability to utilize six hydrocarbons: benzene, toluene, ethylbenzene, m-xylene, o-xylene, and p-xylene. Each strain was standardized to 108 CFU/ml using a 0.5 McFarland turbidity standard and inoculated at 1% into 100 ml of Bushnell Haas broth in a 250 ml airtight conical flask, supplemented with 0.5% ammonium nitrate. After a four-day incubation at 37ºC, we assessed the strains' hydrocarbon utilization by measuring residual hydrocarbons via extraction with hexane and analyzing the results gravimetrically, as per Luna et al. (2009). The total dry weight of the cell biomass was measured by centrifuging the culture and drying the cell pellet at 60ºC.

2.6 Utilization study of hydrocarbons and identification of potential bacteria

The utilization study uses 1% of individual BTEX compounds to calculate cell biomass via a gravimetric method, identifying the potential bacterium.

2.7 Tolerance study of potential bacterium

The study investigated hydrocarbon utilization at 1% concentration, with tolerance levels tested from 1% to 10% for all BTEX compounds, based on cell biomass and residual hydrocarbon concentration.

2.8 Biodegradation activity

The potential strain P. putida HLPSC8 was optimized for maximum biodegradation of hydrocarbons, including benzene, toluene, ethylbenzene, and xylenes. This optimization used a method that varied one parameter at a time, with each parameter standardized to ensure consistence. The process took place in a 250 mL airtight conical flask with a 100 mL working volume, under basal conditions of 1% ammonium nitrate, pH 7, 37°C, and 120 hours of incubation. The inoculum was prepared from the strain in its exponential growth phase, maintaining the same conditions and adjusting the optical density to 0.1, corresponding to 1 × 10^8 cfu/mL.

Biodegradation was measured by analyzing the residual hydrocarbons after extraction with hexane, following the gravimetric method outlined by Yang et al. (2019). All measurements were conducted in triplicate, and results were reported as mean ± standard deviation.

2.9 Standardization of various cultural conditions improving biodegradation activity

Standardizing parameters like pH, temperature, agitation, and nitrogen sources is crucial for enhancing biodegradation. This study examined pH levels from 5 to 9, temperatures between 20°C and 50°C, and agitation from 0 to 400 rpm. Additionally, we assessed nitrogen sources, including peptone, yeast extract, malt extract, beef extract, and ammonium nitrate, at a 1% concentration.

3. Growth kinetics profile over time in relation to biodegradation activities.

The potential strain was studied for its biodegradation activities, focusing on cell biomass formation at regular 12-hour intervals, from the lag phase (0 hours) to the decline phase (168 hours). Evaluations were conducted after separating the cell biomass from the cell-free supernatant by centrifugation at 3000 rpm for 15 minutes. The growth of bacterial cells was estimated by measuring the dry weight of the cell biomass obtained from the centrifuged cell pellets, which were dried in a hot air oven at 50°C for 30 minutes. The residual hydrocarbons were measured directly from the cell-free supernatant using the same gravimetric analysis described earlier (Yang et al.2019).

3.1 GC analysis

At the beginning (0 hours) and end (168 hours) of each hydrocarbon biodegradation study, the cell-free supernatant was analyzed for residual hydrocarbon concentration using gas chromatography-mass spectrometry (GC-MS). The analysis was performed with a Thermo Trace GC Ultra coupled with a Polaris Q mass spectrometer and a TriPlus auto-sampler, utilizing a DB-5 column and helium as the carrier gas.The temperature program increased from 50°C to 250°C at 10°C per minute, maintaining the initial temperature for 2 minutes and holding the final temperature for 10 minutes. The GC flow rate was set at 1 ml/min, resulting in a total run time of 32 minutes. Mass spectra were collected in scan mode (0 to 500 m/z) using electron ionization (EI+) Targeted hydrocarbon analysis was conducted at both time

points (0 and 168 hours), alongside non-targeted analysis. Fatty acids were examined at the end of the biodegradation experiment, and the proportion of specific hydrocarbon degradation was calculated using a specified formula.

$$\% \text{ Hydrocarbon degradation} = \frac{(1 - \text{Peak area of hydrocarbon at 168 hrs}) \times 100}{\text{Peak area of hydrocarbon at 0 hr}}$$

4. Results

4.1 Isolation of hydrocarbon utilizing bacteria

Totally 13 hydrocarbon utilizing bacteria were found with 1 % crude oil as the carbon substrate shown in Table 1. Pure cultures were observed on the basis of colony morphology and Gram staining. Predominant of the strains were found to be Gram-negative rod-shaped bacteria shown in Table 2. All of the pure cultured strains inoculums were standardised and used for screening the potential isolates. The potential hydrocarbon utilizing bacteria were studied by gravimetric and total cell biomass.

Table 1: Cell biomass concentration and crude oil biodegradation activities of phyllosphere bacteria cultured in Bushnell Haas broth

Strains No.	Dry cell biomass (g/L)	Crude oil degradation (%)
PHUB1	0.68	29
PHUB2	1.92	62
PHUB3	0.77	18
PHUB4	0.54	22
PHUB5	1.79	64
PHUB6	0.47	17
PHUB7	2.01	69
PHUB8	1.84	60
PHUB9	0.69	25
PHUB10	0.77	27
PHUB11	1.96	63
PHUB12	0.88	27
PHUB13	0.72	31

*PHUB- Phyllosphere Hydrocarbon Utilizing Bacteria

Table 2: Morphological Characteristics by pure culture isolates

Strains No.	Gram Character	Colony Morphology
PHUB2	Positive /rod shaped	white
PHUB8	Negative/ rod shaped	White

4.2 Screening of potential hydrocarbonoclastic bacteria

Out of 13 isolates 5 isolates such as PHUB2, and PHUB8 found to have great cell biomass ranging from 1.92 g/ L and 1.84 g/L dry cell biomass found to be more hydrocarbonoclastic activity with 10 % crude oil as the carbon substrate by total dry weight of the cell biomass. The crude oil degradation of all the 2 strains found to be 62%, and 60% respectively Table 1.

4.3 Molecular identification of potential strains

All the 2 potential strains of this study were chosen and molecules were identified for further detailed characteristics study by using 16S rRNA sequence. The two isolates such as PHUB2, and PHUB8 were identified as *Bacillus altitudinis* HLPSC2, and *Pseudomonas putida* HLPSC8 respectively.

4.4 Secondary screening of most potential hydrocarbons utilizing bacterium

The utilization study of 2 potential strains were examined individually with 1% of all BTEX compounds. On the basis of gravimetric method and total cell biomass Pseudomonas *putida* HLPSC8 found to be specific in cell biomass ranging from 4.1 to 4.7 g/L and in residual hydrocarbon concentration varying from 22 to 27 % (Fig. 1). Therefore, this strain is considered as potential strain and used for the tolerance study.

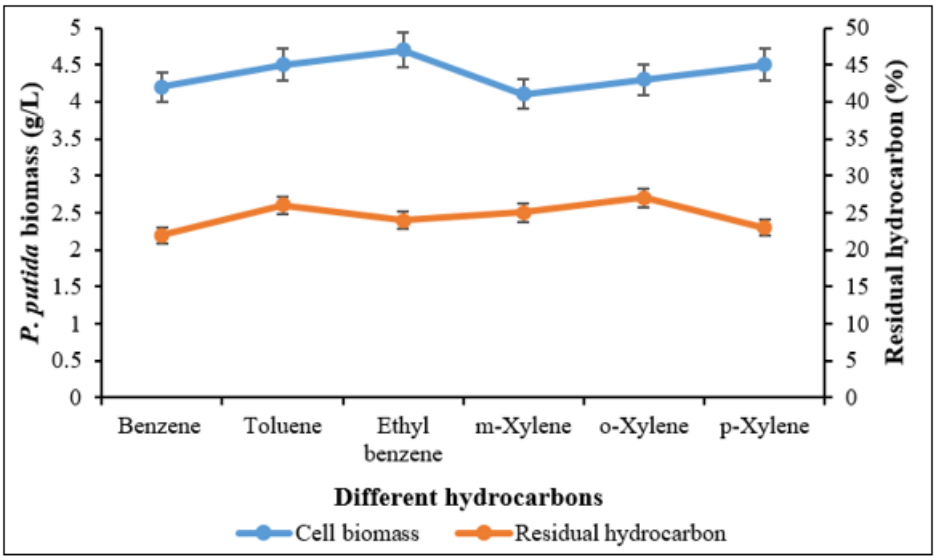

Figure 1: Utilization of different hydrocarbons using *Pseudomonas putida* HLPSC8

4.5 Tolerance study of potential bacterium

The potential *Pseudomonas putida* HLPSC8 strain is used and tolerance study done with BTEX compounds concentration ranging from 1% to 10% see Fig. 2 (a)- (f).

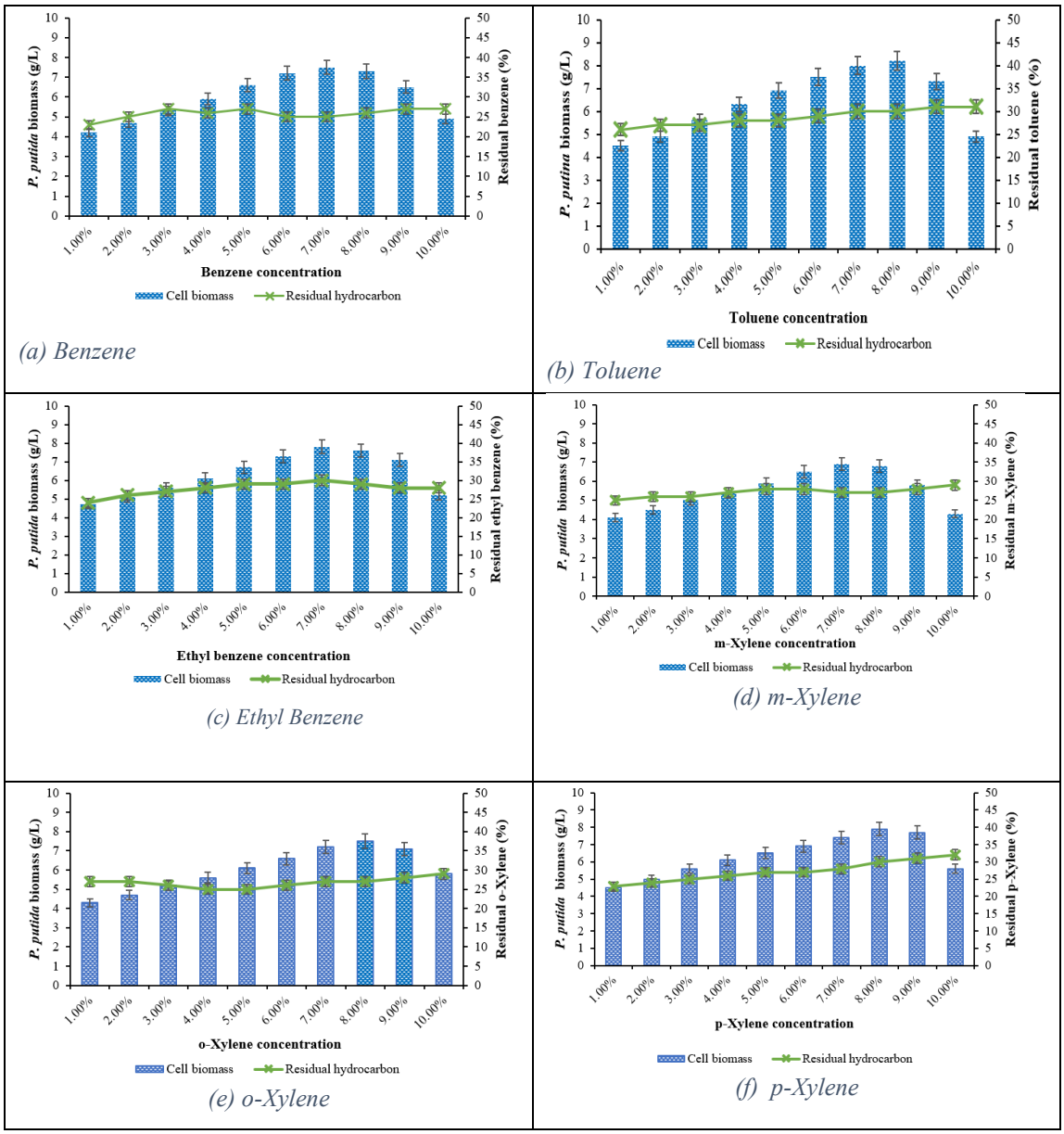

Figure 2: Toleramnce of different hydrocarbons (BTEX) by *Pseudomonas putida* HLPSC8

For Benzene compound Fig. 2 (a), it was found that cell biomass growth increases till 7% and for all other five compounds such as Toluen Ethylbenzene, m-xylene Fig, o-xylene and p-xylene as 8%. see Fig.2 (b)-(f). The residual hydrocarbon concentration found to be 25%, 30%, 29%, 27%, 27% and 30 % for the 6 BTEX compound accordingly.

4.6 Biodegradation activity.

The biodegradation activity of Benzene see Fig. 3(a)-(d) and Toluene see Fig. 4(a)-(d) was analyzed separately by influencing various cultural conditions such as pH from 5 to 9, temperature from 20 to 50 ºC, Agitation from 0 to 400 rpm and different sources of Nitrogen such as Peptone, Yeast extract, Malt extract, Beef extract, Skim milk, Ammonium nitrate, Sodium nitrate and Potassium nitrate. On comparing the cell biomass and the residual hydrocarbon concentration by gravimetric method, it was found that the optimum condition for pH -7, temperature-35º Agitation – 200 rpm and nitrogen source was Ammonium nitrate. This optimum condition helpful in the significant biodegradation for both Benzene and Toluene compounds.

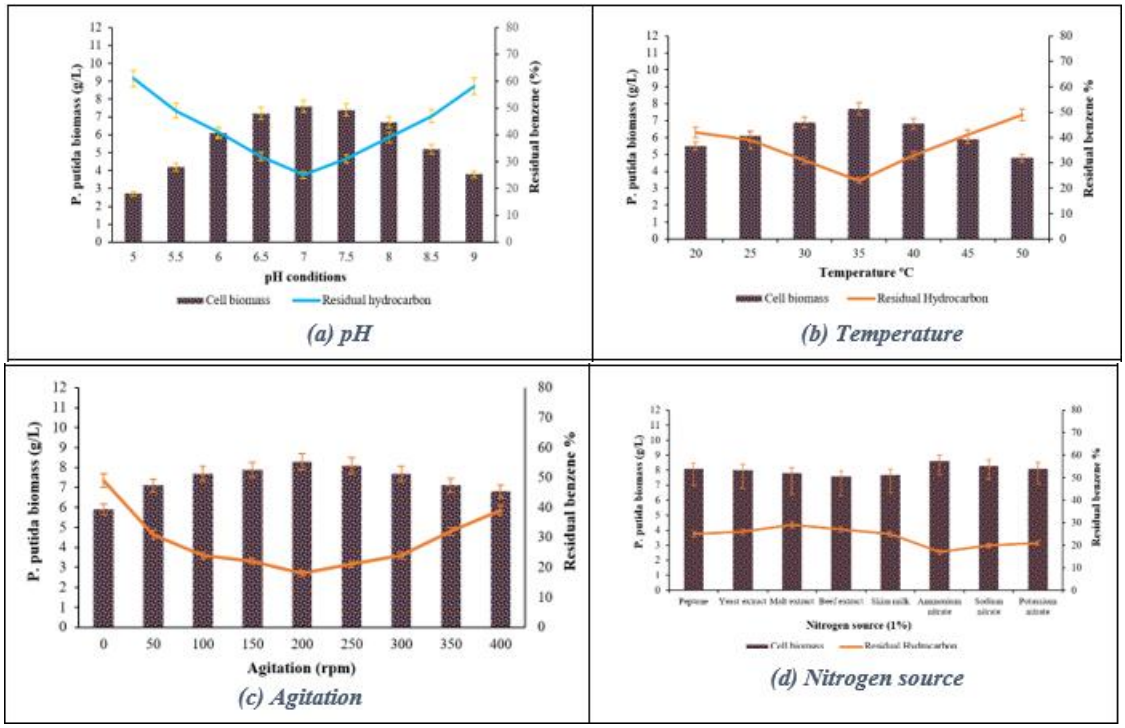

Figure 3: Influence of various factrors in the biodegradation of benzene using *P. putida* HLPSC8

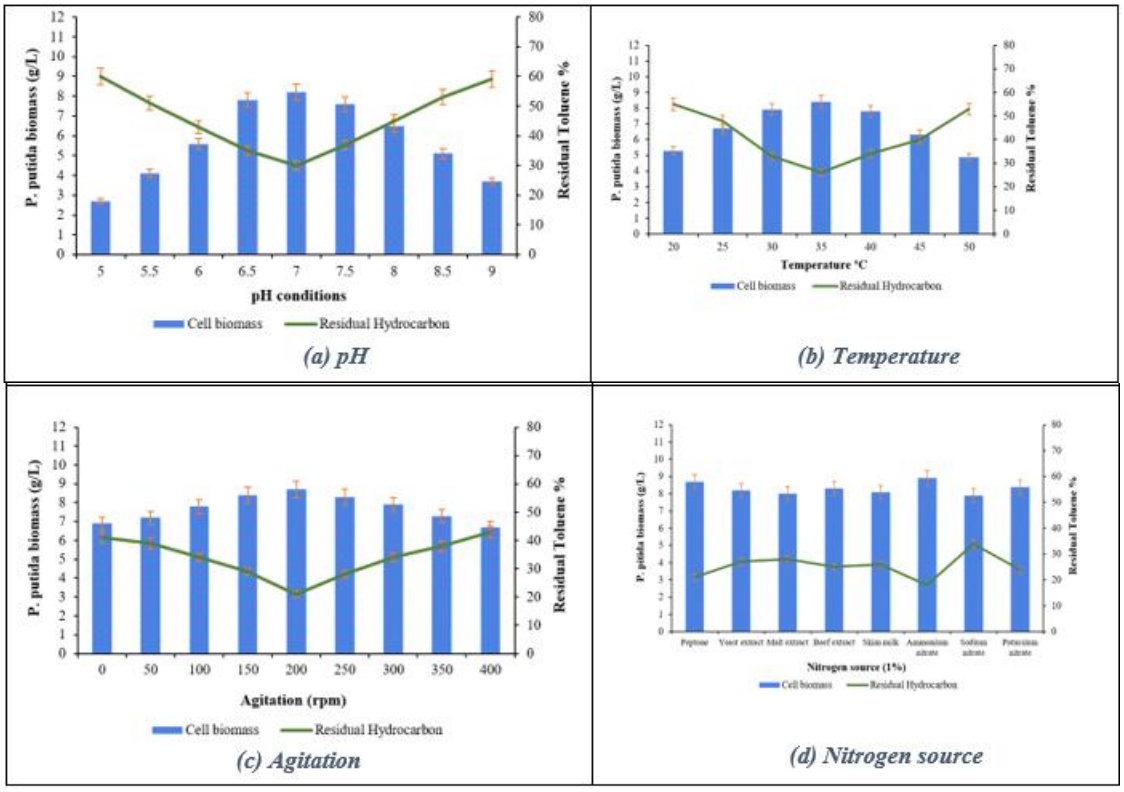

Figure 4: Influence of various factrors in the biodegradation of Toluene using *P. putida* HLPSC8

4.7 Growth Kinetics profile as a function of time on biodegradation activities

The growth kinetics profile over time for biodegradation activities was measured every 12 hours, starting from 0 to 168 hours, for both benzene (Fig. 5) and toluene degradation (Fig. 6).

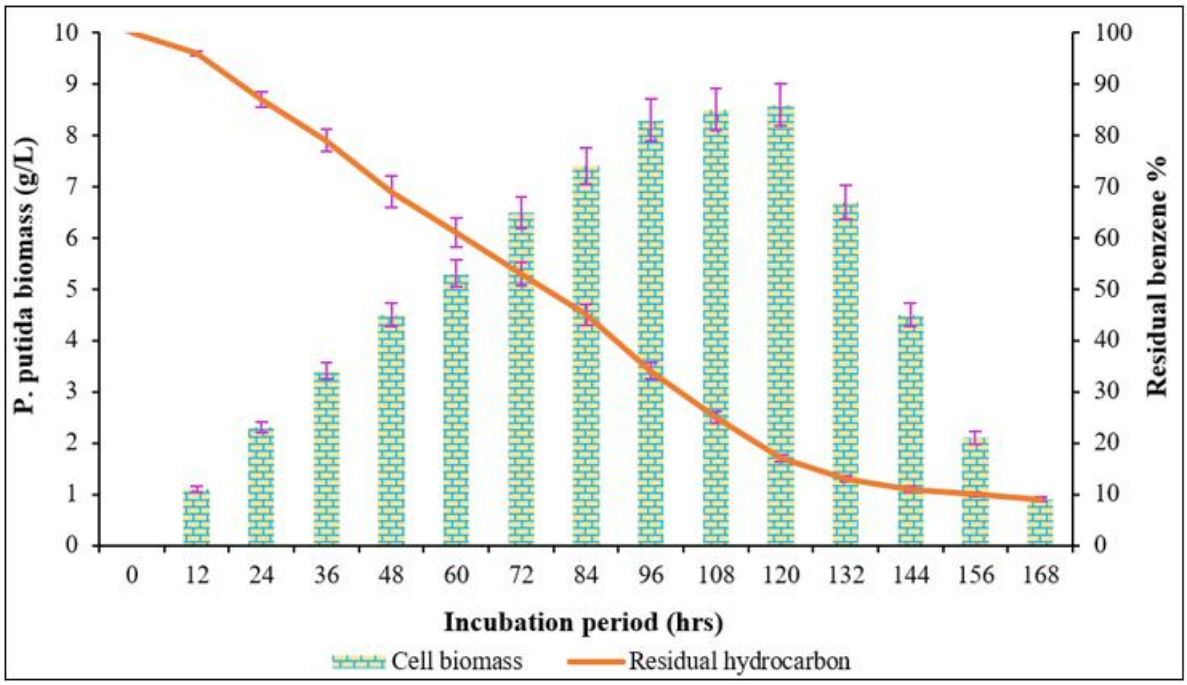

Figure 5: Growth kinetics profile of *P. putida* HLPSC8 with reference to the biodegradation of Benzene

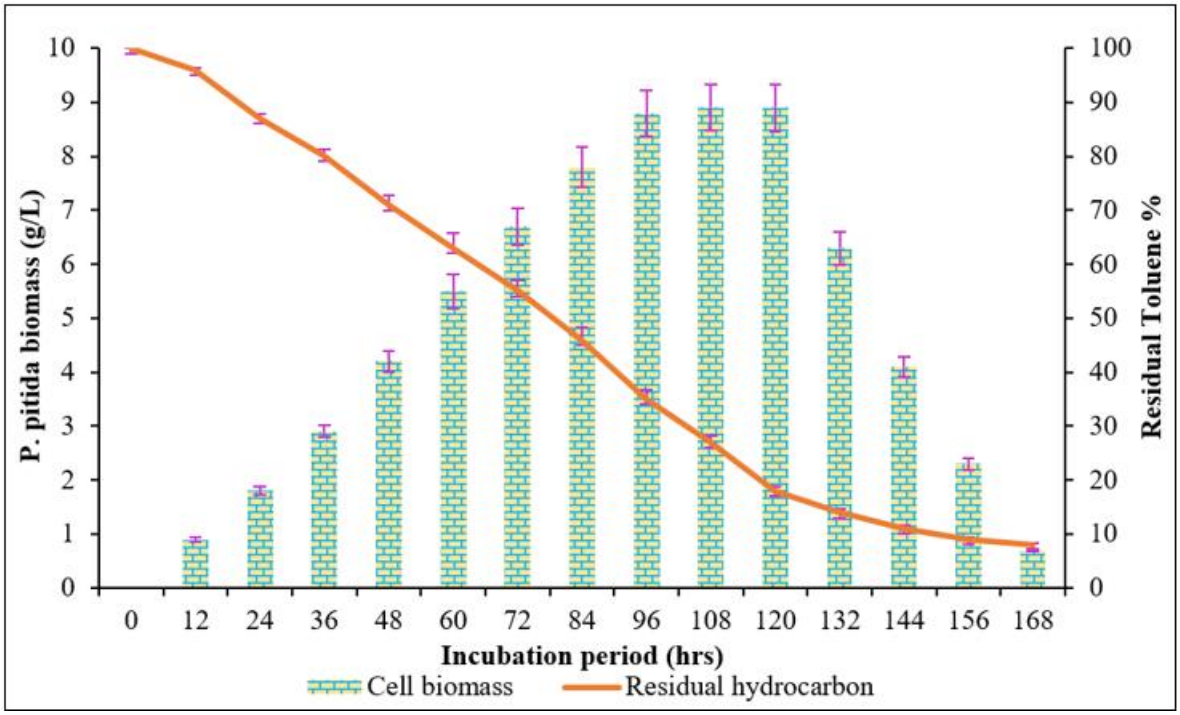

Figure 6: Growth kinetics profile of *P. putida* HLPSC8 with reference to the biodegradation of Toluene

4.8 GC Analysis

The GC analysis was done at the biodegradation broth at 0 hr. and after 168 hr. for targeting the total qualitative analysis of total benzene and toluene. The peak value of Benzene at 0 hr found to be 108862904 and at 168 hrs as 9797661 with the retention time at 19.506 min and 19.361 min respectively see Fig.7 (a)-(b). The peak value of Toluene at 0 hr found to be 120132318 and at 168 hrs as 9610586 with the retention time at 21.981 min and 21.729 min respectively Fig.8 (a)-(b).

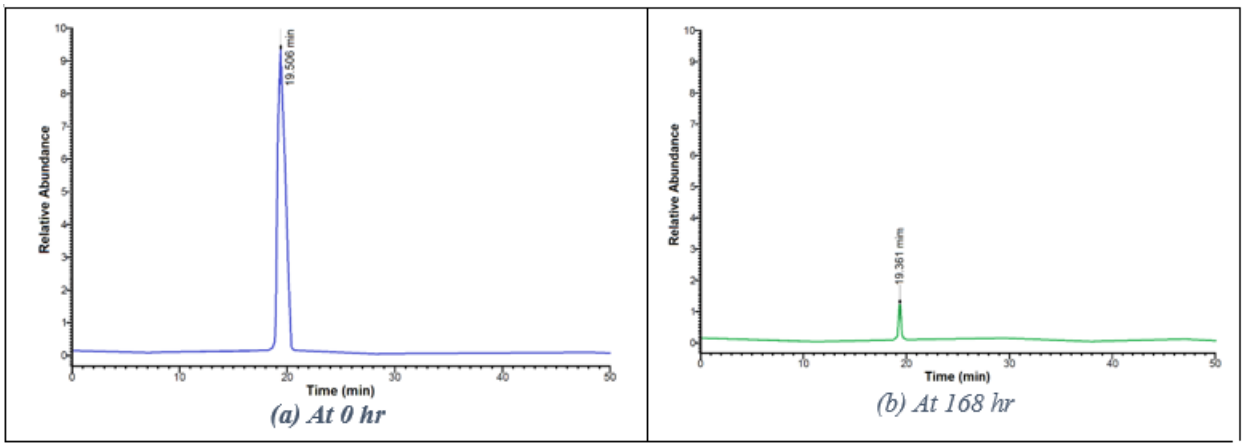

Figure 7: GC analysis of the biodegradation broth after 0 and 168hrs targeting the quantitative analysis of total Benzene

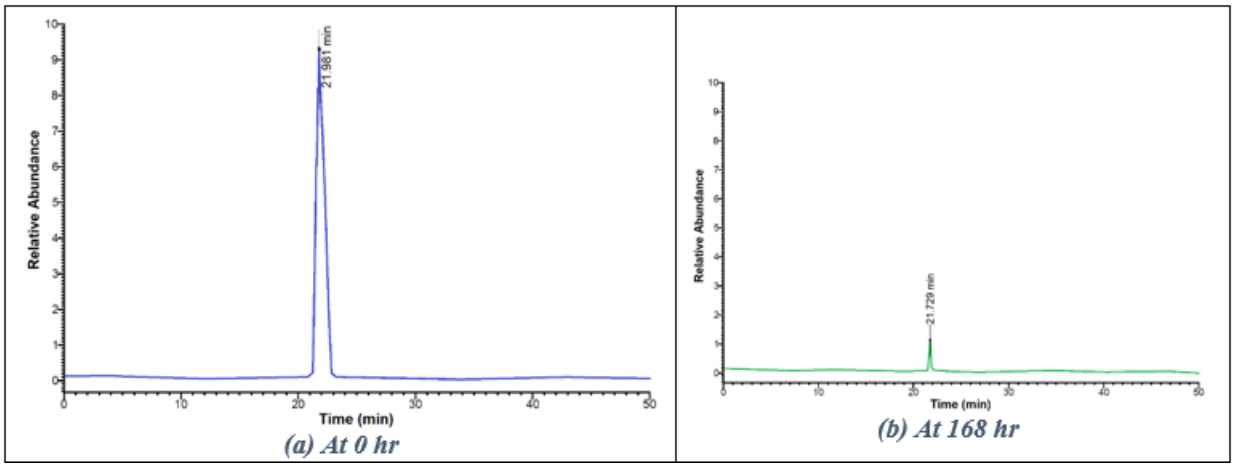

Figure 8: GC analysis of the biodegradation broth after 0 and 168hrs targeting the quantitative analysis of total Toluene

The percentage of biodegradation of both these compounds such as Benzene (Table 3) and Toluene (Table 4) using *Pseudomonas putida* HLPSC8 at the decline phase after 168 hr found to be 91% and 92 % respectively. The GC profile of the biodegradation broth after 144 hr for determining the total volatile compounds was done for both Benzene (Fig 11) and Toluene (Fig 12) compounds. The cell free supernatant after 168 hr and at each retention time shows 15 compounds in benzene (Table 5) and 13 compounds in toluene (Table 6) biodegradation.

Table 3 represents the percentage of benzene biodegradation using *P. putida* HLPSC8 at the end of the experiment (decline phase) after 168 hrs.

Fig no.	Analysis period	Chemical name	Retention time (min.)	Molecular formula and weight	Peak Area
6	0 hrs	Benzene	19.506	C_6H_6, 78	108862904
7	168 hrs	Benzene	19.361	C_6H_6, 78	9797661
Percentage biodegradation of benzene					91%

Table 4: represents the percentage of benzene biodegradation using *P. putida* HLPSC8 at the end of the experiment (decline phase) after 168 hrs.

Fig no.	Analysis perid	Chemical name	Retention time (min.)	Molecular formula and weight	Peak Area
15	0 hrs	Toluene	21.981	C_7H_8, 92	120132318
16	168 hrs	Toluene	21.729	C_7H_8, 92	9610586
Percentage biodegradation of toluene					92%

Table 5: GC analysis of the cell-free supernatant after 168hrs determining the total volatile compounds(Benzene)

S. No	Retention time (min)	Chemical name	Molecular weight	Molecular formula	% Area
1	14.971	2-Butene, 2-methyl-	70	C_5H_{10}	0.9
2	19.379	Benzene	78	C_6H_6	4.6
3	21.678	Propanoic acid, 2-hydroxy-2-	104	$C_4H_8O_3$	7.9
4	22.197	2,3-Pentanedione, 4-methyl-	114	$C_6H_{10}O_2$	1.1
5	24.423	Benzonitrile, 4-ethenyl-	129	C_9H_7N	17.3
6	24.491	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	144	$C_6H_8O_4$	6.7
7	26.803	2-Butynoic acid, 4-cyclohexyl-4-oxo-, ethyl ester	208	$C_{12}H_{16}O_3$	12.1
8	29.084	Benzoic acid, 3,4,5-trimethoxy-	212	$C_{10}H_{12}O_5$	13.9
9	31.100	Heptanoic acid, 3,5,5-triethyl-	214	$C_{13}H_{26}O_2$	22.4

10	31.716	4-Oxazolecarboxylic acid, 4,5-dihydro-2-phenyl-, 1-methylethyl ester	233	$C_{13}H_{15}NO_3$	5.6
11	34.678	Pentadecanoic acid	242	$C_{15}H_{30}O_2$	2.7
12	36.786	2-Pentadecanone, 6,10,14-trimethyl-	268	$C_{18}H_{36}O$	1.2
13	38.817	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296	$C_{20}H_{40}O$	1.3
14	41.629	4-(3,5-Di-tert-butyl-4-hydroxyphenyl) butyl acrylate	332	$C_{21}H_{32}O_3$	0.8
15	43.638	Octadecanoic acid, 2(2hydroxyethoxy)ethyl ester	372	$C_{22}H_{44}O_4$	1.5

Table 6: GC analysis of the cell-free supernatant after 168hrs determining the total volatile compounds (Toluene)

S. No	Retention time (min)	Chemical name	Molecular weight	Molecular formula	% Area
1	15.021	Butanamide	87	C_4H_9NO	0.7
2	17.902	4-Penten-2-ol	86	$C_5H_{10}O$	0.9
3	21.746	Toluene	92	C_7H_8	2.6
4	24.379	2-Cyclopentene-1,4-dione	96	$C_5H_4O_2$	2.7
5	26.896	Propanoic acid, 2-hydroxy-2-	104	$C_4H_8O_3$	3.1
6	29.101	2,3-Pentanedione, 4-methyl-	114	$C_6H_{10}O_2$	1.9
7	29.643	1,3,5-Triazine-2,4,6-triamine	126	$C_3H_6N_6$	13.4
8	31.208	Nonanoic acid	158	$C_9H_{18}O_2$	19.8
9	33.207	2-Naphthalenemethanol, α-methyl-, (±)-	172	$C_{12}H_{12}O$	27.6
10	34.953	Dodecanoic acid	200	$C_{12}H_{24}O_2$	23.1
11	36.488	Heptanoic acid, 3,5,5-triethyl-	214	$C_{13}H_{26}O_2$	1.9
12	39.663	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296	$C_{20}H_{40}O$	1.5
13	43.292	Octadecanoic acid, 2-methyl-, methyl ester	312	$C_{20}H_{40}O_2$	0.8

5. Discussion

On enrichment with Bushnell Haas broth five pure culture were found to be Bacillus altitudinis, Serratia plymuthica, Achromobacter xylosoxidans, Pseudomonas putida and Proteus vulgaris with 1% crude oil as a carbon substrate. Vignesh et al., have isolated microorganisms present in the soil by enrichment technique using Bushnell Haas broth with used engine oil as sole carbon source **(Vignesh et al.2016).**

Though 13 strains isolated from the hydrocarbon-affected leaves with 1% crude oil as the only carbon source, the above mentioned 5 pure cultures found to be potential bacteria. All these strains on gravimetric method **(Luna et al.2009)** showed greater dry cell biomass ranging from 1.79 to 2.01 g/L and degradation capability from 60 to 64 % on study with 10% crude oil extract as a carbon source.

All three methods such as Neighbor Joining tree, Saitou and Nei (1987), UPGMA tree Sneath and Sokal (1973), and Maximum likelihood tree, **Tamura k and Nei M (1993)** were used for understanding the evolutionary relationship all these methods Bacillus altitudinis, Serratia plymuthica, Achromobacter xylosoxidans, Pseudomonas putida and Proteus vulgaris showed 1513,1440,1425,1422 and 1436 position in the final dataset.

On secondary screening of all five potential strain with 1% of individual BTEX compounds the most potential bacteria found to be Pseudomonas putida HLPSC8 and the least one as Serratia plymuthica HLPSC5.You et al. (2013) identified Pseudomonas putida as the maximum biodegradation ability based on the 16S rDNA sequence from hydrocarbon contaminated soil.

On varying concentration of hydrocarbon, the potential strain Pseudomonas putida HLPSC8 found to have significant cell biomass in 7% for Benzene, Ethylbenzene and m-xylene whereas 8% for Toluene-xylene and p-xylene compounds.

The factors such as pH-7, temperature -35 deg.C, Agitation at 200rpm and nitrogen source as ammonium nitrate found to be inclined manner for the biodegradation activity. You et al. (2013) revealed that the biodegradation of the BTEX compounds was greatly influenced by pH, temperature, and salinity.

Gas Chromatography revealed that Pseudomonas putida HLPSC8 found to degrade Benzene at 91% and Toluene at 92% after 168hrs.On the retention time @ 144 hrs. 15 compounds in Benzene and 13 compounds in Toluene biodegradation were found. Rajini Singh and Mary Celin ,2010 on Gas chromatography results reveals that the isolated strain is capable of degrading BTEX in different concentrations ranging 100 % in benzene and 80% in toluene within 48 and 72 hours.

6. Conclusion

Degradation of BTEX is very difficult and only small number of bacterial population were found to degrade in significant manner. The bacterial strains isolated in this study found to be potential and grow in higher concentration of BTEX compounds. Moreover, the degradation of Benzene and Toluene found to be in highest concentration. The derived compounds on degradation may also be used for some other resources and these substrates will prevent the environmental pollution from causing any carcinogenic effects.

References

[1] Amarjyoti Sandhu, Larry J. Halverson and Gwyn A. Beattie (2007), "Bacterial degradation of airborne phenol in the phyllosphere," *Environmental Microbiology*, **9(2),** 383–392 doi:10.1111/j.1462-2920.2006.0114, 9.x.

[2] Andreoni V and Gianfreda L (2007), "Bioremediation and monitoring of aromatic-polluted habitats", *Appl Microbiol Biotechnol* **76,** 287–308

[3] Arenghi, F.L., D. Berlanda, E. Galli, G. Sello, and P. Barbieri (2001), "Organization and regulation of meta cleavage pathway genes for toluene and o-xylene derivative degradation in Pseudomonas stutzeri OX1", *Appl. Environ. Microbiol*, **67(7),**3304–3308.

[4] Calvo.C, M. Manzanera, G. A. Silva-Castro, I. Uad and J. González-López (2009), "Application of bioemulsifiers in soil oil bioremediation processes", *Sci. Total Environ.*, **407**, 3634—3640.

[5] Dean BJ. (1985), "Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols", *Mutat. Res.* **154,**153–181.

[6] Diego A. Hernandez- Ospina , Carlos S. Osorio-Gonz´ alez , Saba Miri and Satinder Kaur Brar (2024), "New perspectives on the anaerobic degradation of BTEX: Mechanisms, pathways, and intermediates ", *Chemosphere**(361)***142490.

[7] Durmusoglu E, Taspinar F and Karademir A (2010). "Health risk assessment of BTEX emissions in the landfill environment", *JHazardMater*,**176.** 870–877.

[8] Eun Jin Choi, Hyun Mi Jin, Seung Hyeon Lee, Renukaradhya K. Math, Eugene L. Madsen,b and Che Ok Jeona (2013), "Comparative Genomic Analysis and Benzene, Toluene, Ethylbenzene, and o-, m-, and p-Xylene (BTEX) Degradation Pathways of Pseudoxanthomonas spadix BD-a59", *Applied and Environmental Microbiology* **(79)** p. 663–671.http://dx.doi.org/10.1128 /AEM.02809-12.

[9] El-Naas MH, Acio JA and El Telib AE (2014), "Aerobic biodegradation of BTEX: Progresses and Prospects", *J Environ Chem Eng,* **2(2),**1104– 1122.

[10] Farhadian M, Vachelard C, Duchez D and Larroche C (2008), "In situ biore- mediation of monoaromatic pollutants in groundwater: a review", *Bioresour Technol,* **99,**5296–5308.

[11] Felsenstein J. (1985). "Confidence limits on phylogenies: An approach using the bootstrap",*Evolution* **39,**783-791.

[12] Hiller, K., Hangebrauk, J., Jäger, C., Spura, J., Schreiber, K. and Schomburg, D (2009), "Metabolite Detector: comprehensive analysis tool for targeted and nontargeted GC/MS based metabolome analysis", *Analytical chemistry*, ***81*(9)**, 3429-3439.

[13] Irwin REA (1997), "Fuel oil number 2 – heating oil entry. In: Irwin RJ (ed) Environmental contaminants encyclopedia. Fort Collins", *National Park Service,* 805–825.

[14] Jindrova E, ChocovaM, Demnerova K and Brenner V (2002) ,"Bacterial aerobic degradation of benzene, toluene, ethylbenzene and xylene", *Folia Microbiol,* **47,**83–93.

[15] Junca H and D. H. Pieper (2004), "Functional gene diversity analysis in BTEX contaminated soils by means of PCR- SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries", *Environ. Microbiol*, 6(2), 95--110.

[16] Kadivar H and Stapleton AE (2003), "Ultraviolet radiation alters maize phyllosphere bacterial diversity", *Microbial Ecology* **45(4),** 353–361, DOI 10.1007/s00248-002-1065-5.

[17] Kahng, H. Y. J. C. Malinverni, M. M. Majko, J. J. and Kukor (2001), "Genetic and functional analysis of the tbc operons for catabolism of alkyl- and chloroaromatic compounds in Burkholderia sp. strain JS150", *Appl. Environ. Microbiol,* **67(10)**, 4805--4816.

[18] Kinkel, L. (1997), "Microbial population dynamics on leaves", *Annu. Rev. Phytopathol. **35**, 327–347.*

[19] Luna, J.M.D., Sarubbo, L. and Campos-Takaki, G.M.D (2009), "A new biosurfactant produced by *Candida glabrata* UCP 1002: characteristics of stability and application in oil recovery", *Brazilian Archives of Biology and Technology*, ***52*,** 785-793.

[20] Maier R. M (2000), "Microorganisms and organic pollutants". *In: Environmental Microbiology (Eds. Maier R.M., Pepper L.I., Gerba P.C.),* **16**, 363-400.

[21] Mohammad BT and Veiga MC, Kennes C (2007), "Mesophilic and thermophilic biotreatment of BTEX-polluted air in reactors", *Biotechnol Bioeng*, **97,**1423–1438.

[22] Monier, J.M., and Lindow, S.E. (2004), "Frequency, size, and localization of bacterial aggregates on bean leaf surfaces", *Appl Environ Microbiol,* **70,** 346–355.

[23] Preethy Chandran and Nilanjana Das (2011), "Degradation of diesel oil by immobilized Candida tropicalis and biofilm formed on gravels", *Biodegradation **22**:1181–1189, DOI 10.1007/s10532-011-9473-1.*

[24] Rahul, Mathur, A.K., Balomajumder, C (2013), "Biological treatment and modeling aspect of BTEX abatement process in a biofilter", *Bioresour. Technol,* **142**, 9–17.

[25] Saitou N. and Nei M. (1987), "The neighbor-joining method: A new method for reconstructing phylogenetic trees", *Molecular Biology and Evolution* **4.**406-425.

[26] Sivasubramanian K, Ravichandran S, Vigneshwaran P (2016), "Isolation of Phenol-Degrading Bacteria from Decapods Mangrove Crabs", *SOJ Microbiol Infect Dis,***4(1),** 1-5.

[27] Sneath P.H.A. and Sokal R.R. (1973), Numerical Taxonomy. Freeman, San Francisco.

[28] Sneath, P. H. A., and R. Sokal. (1973), Numerical taxonomy: the principles and practice of numerical classification. W. H. Freeman, San Francisco, Calif.

[29] Tamura K, Stecher G, Peterson D, Filipski A and Kumar S (2013), "MEGA6: molecular evolutionary genetics analysis version 6.0", *Molecular Biology and Evolution* **30(12),**2725–2729, DOI 10.1093/molbev/mst197.

[30] Tamura K. and Nei M. (1993), "Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees", *Molecular Biology and Evolution,***10,**512-526.

[31] Tamura K., Nei M., and Kumar S. (2004), "Prospects for inferring very large phylogenies by using the neighbor-joining method", Proceedings of the National Academy of Sciences (USA), **101,**11030-11035.

[32] Tamura K., Stecher G., and Kumar S. (2021), "MEGA 11: Molecular Evolutionary Genetics Analysis Version 11", *Molecular Biology and Evolution,* **38(7),** 3022-3027.

[33] Yadav, J.S and Reddy, C.A, (1993), "Degradation of benzene, toluene, ethylbenzene, and xy- lenes (BTEX) by the lignin-degrading basidiomycete phanerochaete

chrysosporium", *Appl. Environ. Microbiol,* **59**, 756–762.

[34] Yang, R., Zhang, G., Li, S., Moazeni, F., Li, Y., Wu, Y., Zhang, W., Chen, T., Liu, G., Zhang, B. and Wu, X (2019), "Degradation of crude oil by mixed cultures of bacteria isolated from the Qinghai-Tibet plateau and comparative analysis of metabolic mechanisms", *Environmental Science and Pollution Research*, ***26***, 1834-1847.

[35] You Y, ShimJ, Cho CH, RyuMH, Shea P, Kamala-Kannan S, Chae JC, Oh BT (2013), "Biodegradation of BTEX mixture by Pseudomonas putida YNS1 isolated from oil-contaminated soil", *J BasicMicrobiol*, **53,**469–475.

[36] Zhang, Y., Luo, X., Wang, X., Qian, K., and Zhao, R. (2007), "Influence of temperature on formaldehyde emission parameters of dry building materials", *Atmos. Environ,* **41**, 3203–3216. http://dx.doi.org/10.1016/j.atmosenv.2006.10.081