Tolerance and Biodegradation of Benzene, Toluene, Ethylbenzene and Xylenes (BTEX) by a Metal Acclimatized Bacterial Consortium Culture

Fellie E. A.,[1] Sannasi P., *[2] Wong K. K.,[1] Salmijah S.,[1] and Kader J.[3]
1. Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor D.E., MALAYSIA
2. Faculty of Health and Life Sciences, INTI International University, 71800 Nilai, N. Sembilan, MALAYSIA
3. Faculty of Science and Technology, University Sains Islam Malaysia, 71800 Nilai, Negeri Sembilan, MALAYSIA
*palsan.abdullah@newinti.edu.my

Abstract

This investigation reports the tolerance and biodegradation of benzene, toluene, ethylbenzene and xylene isomers (BTEX) by a heavy metal-adapted environmental bacterial consortium, known as consortium culture (CC). Higher tolerance was observed with benzene (IC_{50} value up to 191.25 mg/L), followed by toluene (IC_{50} = 139.67 mg/L), xylene (IC_{50} = 97.04 mg/L) and ethylbenzene (IC_{50} =96.99 mg/L). Significant decrease ($p < 0.05$) in the specific growth rate (μ), however was observed as the concentrations of each individual BTEX were increased from 10 mg/L to 500 mg/L. Growth of CC was completely inhibited at 250 mg/L ethylbenzene and 500 mg/L xylene. Toxicity followed the trend: B<T<X<E. Biodegradation of individual BTEX compound was monitored by gas chromatography. The GC-FID chromatographic profiles showed the capability of CC to significantly biodegrade ($p < 0.05$) benzene (61.66 %), toluene (55.91 %), ethylbenzene (37.15 %), p-xylene (43.66 %), m-xylene (47.86 %) and o-xylene (41.03 %) at an initial concentration of 50 mg/L after 48 hours. These findings confirm the ability of CC to withstand biodegrade and utilize BTEX as the sole source of carbon and energy in the following order: B>T>X>E.

Keywords: BTEX, BTEX tolerance, bacteria, bioremediation, biodegradation.

Introduction

The extent of crude oil and petroleum-originating hydrocarbon contamination has been extensively discussed and reviewed over the years. Although the biodegradation of crude oil has been much emphasized; the removal of benzene, toluene, ethylbenzene, and xylene isomers, collectively known as BTEX still poses many questions. BTEX are volatile organic compounds and are ubiquitous in nature as constituents of fuel i.e. gasoline, diesel and aviation fuel, in insecticides, plastics, dyes, furniture wax, paints and paint thinners, or used as polymer adhesives and solvents, amongst others and have been classified as priority pollutants as BTEX are known carcinogen and neurotoxin with prolonged continuous exposure [1]. Its increased use has inevitably contributed to terrestrial water as well as groundwater pollution[2] as BTEX easily migrate through groundwater systems due to their increased solubility and mobility and able to bioaccumulate through the food chain. Accidents, spills during transport, leakage from underground storage tanks, or from waste disposal sites, surface and storm water run-off, and leaching landfills further contribute to this problem[3]. Available physico-chemical approach such as volatilization, adsorption, chemical oxidation, photo-decomposition, steam injection and electrical heating pump and treat method, vapor extraction require more effort, energy and cost intensive.

Biocatalytic activity of hydrocarbon degrading enzyme can be inhibited by the presence of other environmental pollutants such as heavy metals[4, 5]. Any remedial action has to be taken into account. Biodegradation of BTEX using pure bacterial culture such as *Pseudomonas* species has been extensively studied[6, 7]. Biodegradation of complex hydrocarbons usually requires the cooperation of more than species or a consortium of bacterial culture[8]. Theoretically, each microbe utilizes hydrocarbon in a distinctive pathway and relies on the existence of other species or strain to survive given that energy source is limited in the presence of complex hydrocarbon in the microbial soil[9].

The consortium culture (CC) used in this study is sourced from metal-contaminated sites with good Cr(VI), Cu(II), and Pb(II) metal loading[10] and metal removal capability[11]. Furthermore, coupled to the fact that these isolates are heavy metal tolerant, the ability of these microbes to withstand organic solvents presents an enormous advantage and possibility of harvesting enzymes and protein products of importance for the biotechnology industry. The objective of this study was to investigate the effect of BTEX towards CC and the capability of CC to biodegrade BTEX and use it as their sole source of carbon and energy.

Materials and methods

Source of bacterial culture: The metal acclimated consortium culture (CC) consists of 6 gram negative bacteria (*Agrobacterium sp., Chryseomonas sp., Flavobacterium sp., Pseudomonas sp., Serratia sp.* and *Xanthomonas sp.)* and 3 gram positive bacteria (*Arthobacter sp., Bacillus sp.* and *Micrococcus sp.)*[10, 12] from prior glycerol stock culture (at -80° C) and thawed at room temperature. Cell biomass was separated by centrifugation (4000 rpm, 10 min); pellets were washed in

normal saline (0.85 % NaCl), re-centrifuged and rinsed twice before re-suspended in 10 mL saline. Bacterial growth was monitored by optical density (OD) at 600 nm (spectrophotometer; Hitachi U1100, Japan); plate counts as colony forming units/mL (cfu/mL) and correlated dry weight (g) of biomass[10].

Culture medium: Difco™ Bushnell-Hass (BH) media containing (per L) 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1.0 g KH_2PO_4, 1.0 g $(NH_4)_2HPO_4$, 1.0 g KNO_3 and 0.05 g $FeCl_3$ was used. Media solution was autoclaved at 121°C for 15 min. and cooled to room temperature before inoculation of bacteria. One percent (v/v) BTEX was used as the sole carbon source to acclimatize the cells. All steps involving addition of BTEX into the media was carried out at 4°C to prevent evaporation of the volatile BTEX. Media refreshment was carried out fortnightly. Test values obtained were deducted by values measured in control sets to substantiate for abiotic BTEX losses.

Starter inoculum preparation: The suspended pellet is inoculated to 100 mL of BH media containing 1% (v/v) BTEX and incubated at room temperature (28°-30°C) on an orbital shaker (150 rpm; Thermo Scientific, USA) until it attains OD_{600} = 0.5. Cell counts per mL were determined by the use of hemacytometer. An inoculum size of 5% (v/v) standardized to cell density of 0.5 at OD_{600} (containing approx. 10^7 cells/mL) is usedfor subsequent tests[10, 12].

Hydrocarbons and chemicals: Benzene (B), Toluene (T), Ethylbenzene (E) and Xylene isomers (o-X, m-X, and p-X) were obtained from Sigma-Aldrich Chemical Co. (UK). All the chemicals used are of reagent grade.

Tolerance level of BTEX- lag phase time and specific growth rate: The ability of CC to tolerate BTEX individually is ascertained by observing the time needed for the culture to acclimatize and adapt to the hydrocarbon substrates. Specific growth rate and initial lag phase time determination experiment were carried out to determine the effect of different concentrations of BTEX towards CC's growth. Individual BTEX compound was added into BH media to obtain a final concentration of 10, 50, 100, 250 and 500 mg/L. The culture was inoculated with 5% (v/v) CC aliquots. Final volume was 100 mL. Incubation was carried out at room temperature on a rotary shaker (150 rpm) for 48 hours along with suitable controls. Tests were done in triplicates. Three mL aliquots were withdrawn periodically from each bottle at 4 h intervals for 48 h under aseptic conditions, and cell concentrations at 600 nm were determined. The initial lag period and point of time as to when growth starts to peak (i.e. initial lag time) was noted. Specific growth rate, μ (h^{-1}) was determined according to eq. (1)[13]:

$$\text{Specific growth, } \mu = \ln (X - X_0) / (t - t_0) \quad (1)$$

where μ is specific growth rate (h^{-1}); X is no. of cells (cell/mL) at the end of exponential phase; X_0 is no. of cells (cell/mL) at the beginning of exponential phase; t is time point (h) for [X] and t_0 is time point (h) for [X_0].

Inhibitory concentration (IC_{50}) of BTEX: The inhibitory concentration of individual compounds of BTEX towards CC was performed at concentrations of 10, 25, 50, 80 and 100 mg/L. Five mL aliquot of CC (5%, v/v) was added to 100 mL of BH media spiked with individual BTEX as the sole carbon source. Culture bottles were agitated at 150 rpm for 24 h at room temperature (28°-30°C). The cell concentration at 600 nm was determined using a spectrophotometer (Genesys, USA) at 0 h (initial) and after 24 h. Cells grown in nutrient broth (NB) served as control (without hydrocarbon) to observe the maximum growth of CC attained and was considered as 100% growth. The effect of individual BTEX compound was reported by ascertaining the maximal inhibitory concentration deemed to reduce bacterial population growth by half; i.e. inhibitory concentration (IC_{50}). If the growth falls below 50%, that particular tested concentration level is deemed to be toxic and inhibitory to CC (lower tolerance). Growth inhibition (I, %) was calculated using the following:

$$\text{Growth inhibition (I, \%)} = (C - N / C) \times 100 \quad (2)$$

where C is no. of cells (cells/mL) in NB (control; without BTEX) after 24 h and N is no. of cells (cells/mL) in BH (test; with BTEX) after 24 h.

BTEX biodegradation study conditions: Individual BTEX compound was added into a 40 mL vial containing 28.5 mL of BH media to obtain a final concentration of 50 mg/L via polytetrafluoroethylene (PTFE)/silica septum (11 mm × 1.9 mm, Supelco, USA). A CC aliquot of 1.5 mL prepared as above was then pipetted into the vial. Culture vials were incubated at room temperature and agitated at 150 rpm. Samples (3 mL) were withdrawn from each vial using a gas-tight syringe (Hamilton, USA) at 0, 24 and 48 h of incubation and kept in a cooled (4°C) 4 mL vial. One mL sample is used for CC's cell count and the remaining 2 mL for headspace BTEX residual extraction using solid phase micro-extraction (SPME) method.

Residual BTEX extraction: Solid phase micro-extraction (SPME) fiber (Supelco, USA) coated with 100 µm polydimethylsiloxane (PDMS) was used to extract and trap residual BTEX from samples[14]. Samples were stirred vigorously at room temperature and the fiber was exposed to the sample for 4 min in the headspace to allow maximal adsorption of BTEX residues following which the trapped hydrocarbon in the fiber is injected into the GC and left for 3 min for desorption.

Chromatographic analyses of residual BTEX: Residual BTEX was quantified using GC-FID (Hewlett Packard HP 5890) equipped with a capillary column HP-5 (30 m × 0.32 mm × 0.25 µm). Helium was used as the carrier gas with a

flow rate of 2.3 mL/min. The temperature of the injector and detector was set at 150°C and 320°C respectively. The column temperature was initially programmed at 50°C, held for one minute, then increased at 20°C/min to 90°C (held for 1 minute), and later to 120°C (held for 10 minutes). BTEX biodegradation by CC was evaluated based on peak reduction observed on the chromatogram profile (Eq. 3). Peak area reduction in test samples was corrected with control sets to account for abiotic losses.

$$\text{Degradation \%} = [(PA_0 - PA_n) / PA_0] \times 100 \quad (3)$$

where PA_0 is the peak area observed at time 0, and PA_n is the peak area at any specified time intervals.

Statistical analysis: Experimental data were subjected to statistical analysis for mean tests, linear correlation and least squares regression by Excel (Office 2007) and t-tests and one-way analysis of variance (ANOVA) by MINITAB v. 13.2 (Minitab Inc., USA). Significant levels were set at $\alpha = 0.05$.

Results and Discussion

Tolerance level of BTEX- initial lag phase time and specific growth rate: Figure 1 and table 1 present the effect of individual BTEX at varying concentrations (10-500 mg/L) in terms of initial lag phase time and specific growth rate (μ, h^{-1}). Cell growth (cells/mL) obtained was used to calculate specific growth rate, μ (h^{-1}) to describe the phenomenon observed during 48 h of incubation in comparison to control cultures. The cell counts generally increase after 8 to 12 h with all BTEX compounds at concentrations up to 50 mg/L and although specific growth rate dropped (in the range of 0.11-0.14 h^{-1}), the reduction was not significant ($p > 0.05$). Specific growth rate showed significant decrease at 50 mg/L (with xylene isomers) and at 100, 150, 250 mg/L with all the tested compounds ($p < 0.05$). The longest initial lag time was observed with toluene (at 500 mg/L) up to 32 h. While a longer time is required for growth to pick up as concentrations are increased, it was apparent that BTEX are consumed by CC as their sole source of carbon and energy. Interestingly, benzene and toluene supported CC growth at the highest concentration level tested i.e. 500 mg/L.

Shim et al[6] have indicated that benzene at 500 mg/L completely inhibited the growth of a co-culture mixture consisting of *P. putida* and *P. fluorescens*. Among the tested hydrocarbons, benzene had the least effect overall as seen from the higher and positive specific growth rate of 0.14 h^{-1} (at 10 mg/L), 0.11 h^{-1} (at 50 mg/l), 0.10 h^{-1} (at 100 and 250 mg/L) and 0.06 h^{-1} (at 500 mg/L) when compared to control cells (0.20 h^{-1}). The CC was highly tolerant in benzene; followed by toluene and xylene. At 500 mg/L, both ethylbenzene and xylene isomers were highly toxic as growth was completely inhibited. Xylene however was less toxic with μ of 0.05 h^{-1} at 250 mg/L compared to ethylbenzene (complete inhibition). This suggests the toxic nature of specific BTEX compounds to CC's cell growth particularly at higher concentrations affects their relative tolerance. Our observations imply that each hydrocarbon differs in degree of toxicity amid the ability of CC to utilize them to sustain growth. Ethylbenzene was most toxic to CC's growth followed by xylene, toluene and benzene.

Inhibitory concentration (IC_{50}) of BTEX: To support the above observation, IC_{50} reading was determined to ascertain the cytotoxicity effect of varying concentration of BTEX compound. This can be used as an indicator to estimate overall tolerance and toxicity of BTEX to CC, as one can estimate as to what concentration (mg/L) BTEX starts to inhibit bacterial growth. Figure 2 shows the degree of inhibition (I, %) of CC's growth after 24 h of incubation with exposure to different BTEX concentration. In general, growth of CC was significantly affected by BTEX concentrations as observed from the higher percentage of inhibition (I, %) as BTEX concentration was gradually increased from 10 mg/L to 150 mg/L (Figure 1).

Furthermore, individual BTEX compound can exert varying effect of toxicity towards CC's growth. Through this data, the minimal inhibitory concentration (IC_{50}) of individual BTEX compound was ascertained. Through the use of linear regression equation ($R^2 > 0.95$, Figure 2), ethylbenzene was shown to be most toxic to CC with the lowest IC_{50} value of 96.99 mg/L, followed by xylene (IC_{50} = 97.04 mg/L), toluene (IC_{50} = 139.67 mg/L) and benzene (IC_{50} = 191.25 mg/L). Complete inhibition of CC growth by benzene is predicted at 374.87 mg/L, followed by toluene (273.11 mg/L), xylene (206.97 mg/L) and ethylbenzene (196.97 mg/L). Toxicity of BTEX to CC was found to be E>X>T>B.

BTEX degradation by consortium culture: To investigate the extent and ability of CC to biodegrade BTEX compounds, CC was exposed to 50 mg/L of BTEX individually for 48 hours in BH media. This concentration level was chosen and standardized for all the BTEX compounds based on the IC_{50} value obtained. Residual BTEX from the culture sets were quantified after 24 and 48 h using GC-FID. Analysis was done semi-quantitatively by calculating the peak area of BTEX residues on each chromatogram profile. Abiotic control was used to correct for BTEX loss due to non-biological factors. Fig. 3 shows biodegradation of BTEX after 24 and 48 hours of incubation.

After 24 hours, benzene biodegradation was observed to be the highest (35.43 %, $p < 0.05$), followed by toluene (29.55 %), m-xylene (30.00 %), p-xylene (29.38 %), o-xylene (24.21 %) and ethylbenzene (21.41 %). As incubation period was extended to 48 h, biodegradation percentage ascended i.e. increased 1.7 times for benzene (61.66 %), up to 55.91 % with toluene, m-xylene (47.86 %), p-xylene (43.66 %) and o-xylene (41.03 %), while biodegradation of ethylbenzene was up to 37.15 %. In overall, the metal-acclimated CC was able to utilize BTEX compounds for

growth and benzene was biodegraded more rapidly followed by toluene, xylene isomers and ethylbenzene (B>T>X>E).

The results demonstrate the capability of CC consisting of mixed gram positive and gram negative isolates to live and biodegrade BTEX compound. The presence of heavy metals together with the organic pollutants will influence the efficiency of biodegradation. This will be advantageous in wastewater treatment, for example the favorable simultaneous biodegradation of phenol and Cr(VI) reduction observed with environmental bacterial isolates previously[5]. As many studies have focused with defined single cultures, now a days more research is geared towards application working with mixed bacterial cultures and mixed wastes. When a single culture of *S. maltophilia* was used, BTEX biodegradation was limited to benzene, toluene and ethylbenzene but not xylene[15]. Similarly, single culture of the fungus *Cladophialophora* was only able to degrade toluene and ethylbenzene but not benzene and xylene[16]. The degradation of a variety of organic compounds such as hydrocarbons requires specific biocatalytic enzyme produced by many different bacteria. Bacterial degradation of aromatic compounds involves oxidative attack by ring cleavage dioxygenases[8, 9].

When a mixed culture is used, more enzymes can be sourced to facilitate and enhance the degradation process. Several studies have shown the capabilities of *Pseudomonas* species to biodegrade petroleum hydrocarbons such as benzene, toluene, ethylbenzene and xylene as they harbor specific catabolic genes in biodegrading hydrocarbon BTEX via a few pathways both aerobically and anaerobically[7]. *Pseudomonas* species is also one of the constituent of CC and suggests the likelihood of it to be actively involved in the catabolic activity of BTEX. The use of CC as in this study will therefore enhance the overall efficiency in biodegrading hydrocarbons such as BTEX or other complex hydrocarbons. In addition, CC is known to produce exopolymers[12] which can form biosurfactants in stressed environmental conditions. Biosurfactants reduce surface tension of the media and increase hydrocarbon solubility. This enhances bioavailability of the hydrocarbon for ease of biodegradation by the bacterial cells.

From the chemistry perspective, toxicity effect exerted by the individual BTEX compounds can be attributed to their chemical structure. For example, benzene consisting of 6 carbon atoms fused into a ring structure is less toxic than toluene which is a benzene ring with one additional methyl group ($-CH_3$) on its ring. Xylene contains two methyl groups on its benzene ring while ethylbenzene, on the other hand has a longer alkyl group ($-CH_2CH_3$) attached to its benzene ring. Studies on the effect of chemical structure on chemolithotrophic bacteria showed that the increase in size and number of alkyl groups in a benzene ring increases the toxicity of the compound[17]. Similarly, the present study showed that ethylbenzene with an addition of ethyl group is most toxic followed by xylene isomers (two methyl groups), toluene (one methyl group) and benzene (no methyl group), and the oxidation of the ethyl group in ethylbenzene prior to the degradation of its benzene ring was demonstrated[18]. Furthermore, the addition of an alkyl group on the benzene ring can either inhibit or reduce the degradation rate of the hydrocarbons[19].

Therefore it can be concluded that hydrocarbons which are of low toxicity allow the sustained growth of bacterial cells and with time improve and support the bacterial capability to degrade the hydrocarbons. It is found in this study that ethylbenzene having an ethyl group as the additional group is least degraded, followed by xylene with two methyl group, toluene with one methyl group and lastly benzene. Thus results express that degradation of BTEX depends on the length and number of alkyl group present on its benzene ring. In addition, the lower solubility of ethylbenzene and xylene isomers (0.20 g/L) as opposed to benzene (0.7 g/L) and toluene (0.5 g/L) would have limited their bioavailability as substrates. A point to note, in order to utilize or grow on substrates of low solubility's in water, microbes display some physiological adaptations, for example the formation of emulsifiers or biosurfactants to enhance the bioavailability of these hydrophobic compounds in order to utilize them as potential carbon and energy source[3] as microbial growth on hydrocarbons have been linked to their capacity of producing polymers with surfactant activity.

Conversely, benzene and p-xylene were found to be more toxic under anaerobic conditions[20]. In this study, biodegradation was the highest with B>T>m-X>p-X>o-X>E which concurred with the level of toxicity. The ability of CC to degrade individual BTEX was found to be influenced by the level of tolerance and growth inhibition experienced by CC. A hydrocarbon that is less toxic is observed to be more readily degradable. Benzene, for instance as the least toxic of the BTEX compounds was biodegraded the most by CC (35.43 % by 24 h and 61.66 % by 48 h) as opposed to the other hydrocarbon compounds. The reduced degradation rate of hydrocarbon may also depend on the on the aspects of pathways and mechanism of the biodegradation process[9]. However, the mechanisms of individual biodegradation of BTEX and routes followed are not reported here.

Conclusion

In conclusion, results confirm that CC is able to grow and withstand the toxic nature of individual BTEX to a relatively high concentration albeit going through a period of adaptation and acclimatization. Benzene was found to be less toxic to CC, followed by toluene, xylene and ethylbenzene. In addition, CC was shown to biodegrade individual BTEX compounds up to 41-61% after 48 h thus affirming that BTEX compounds are consumed as their sole source of carbon for energy and cell growth. This

findings favors for the application of CC in the bioremediation of sites contaminated with BTEX petroleum hydrocarbons and supports the development of an integrated bacterium-based priority pollutant waste treatment system.

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Table 1
The effect of individual BTEX compounds towards CC growth over 48 h of incubation

Concentration, mg/L	Initial lag phase time, h	Specific growth rate (μ), h^{-1}
Benzene		
Control	8	0.20
10	8	0.14
50	12	0.11
100	12	0.10*
250	12	0.10*
500	28	0.06*
Toluene		
10	12	0.12
50	12	0.11
100	12	0.10*
250	16	0.08*
500	32	0.05*
Ethylbenzene		
10	12	0.11
50	12	0.11
100	16	0.07*
250	NG	NG
500	NG	NG
Xylene isomers		
10	8	0.12
50	12	0.08*
100	12	0.06*
250	28	0.05*
500	NG	NG

Values are mean of triplicates; NG = no growth* = significantly different at α = 0.05

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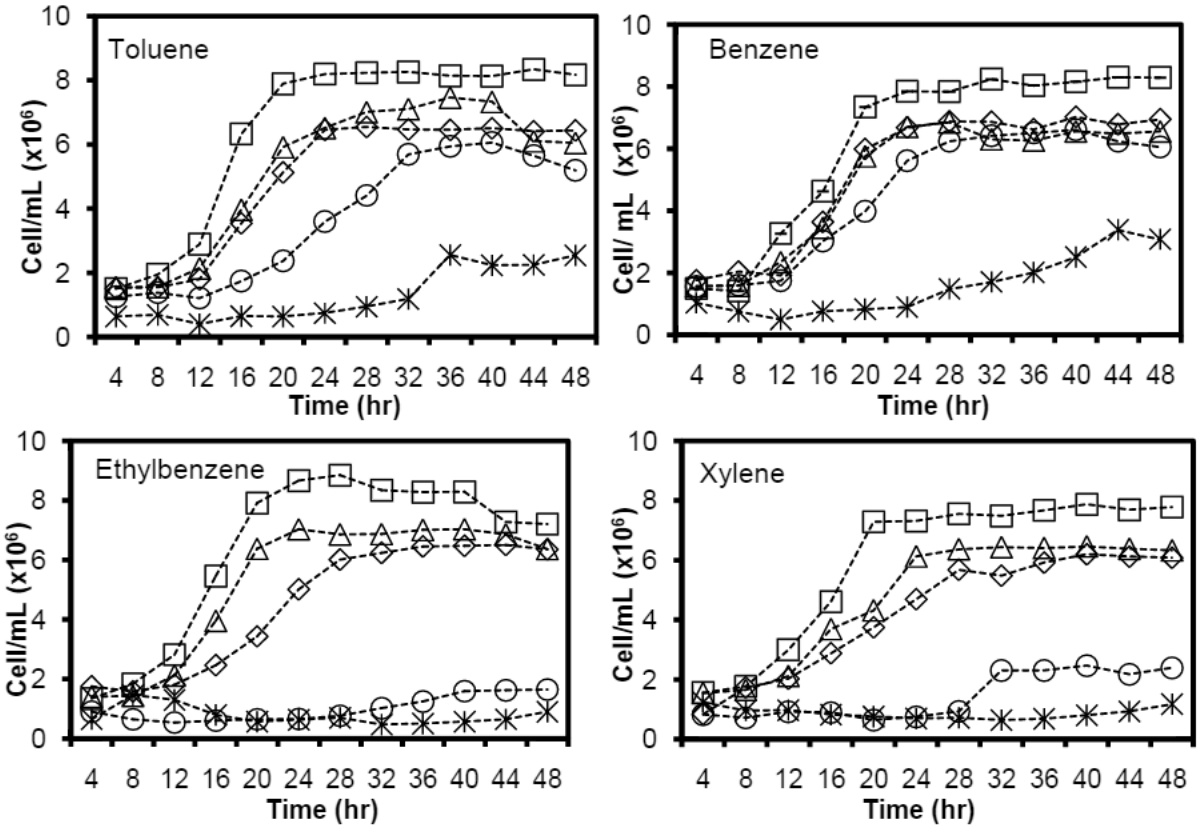

Fig. 1: Growth response of CC towards individual BTEX at 10 mg/L (-□-), 50 mg/L (-△--), 100 mg/L (··◇··), 250 mg/L (-○--) and 500 mg/L (·✳··) during 48 hours incubation with agitation at 150 rpm in room temperature. All test bottles contain starting concentration of 5% (v/v) CC.

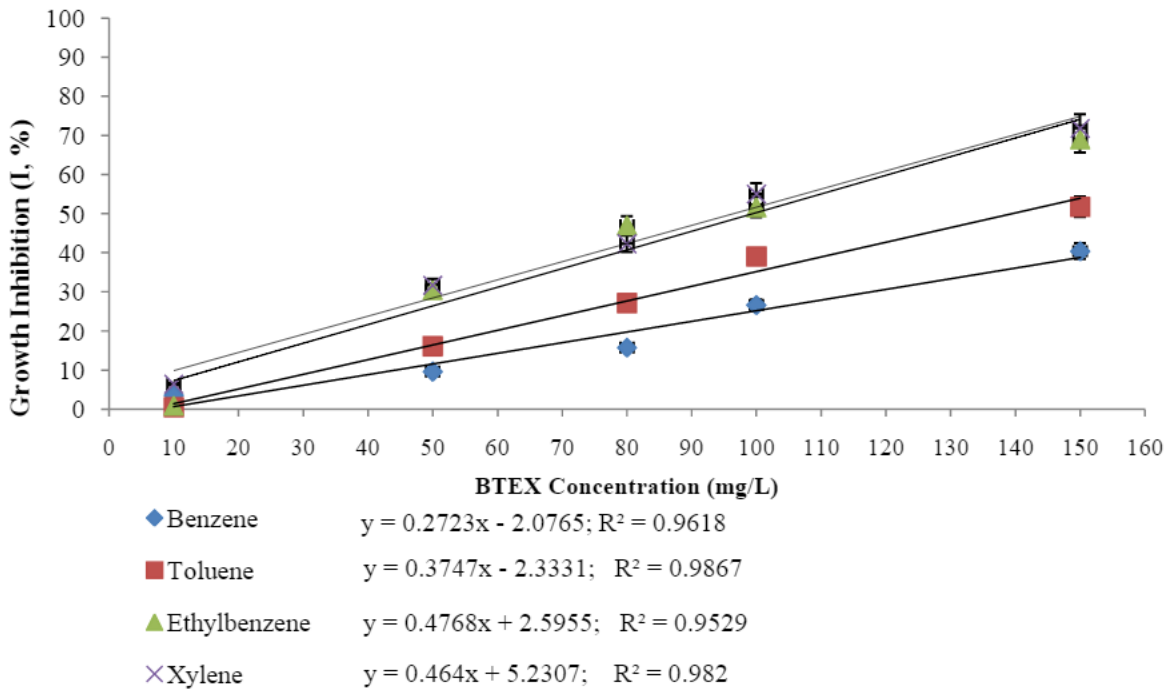

Fig.2: Growth inhibition (I, %) of CC exposed to benzene (-◆-), toluene (-■-), ethylbenzene (-▲-), and xylene isomers (-×-) after 24 hours of incubation at room temperature. Linear regression equation are shown for each BTEX compound and used to compute IC_{50}. Test bottles contain 5% (v/v) CC exposed to 10, 25, 50, 80 and 100 mg/L of BTEX individuals. Values are mean ± s.e.

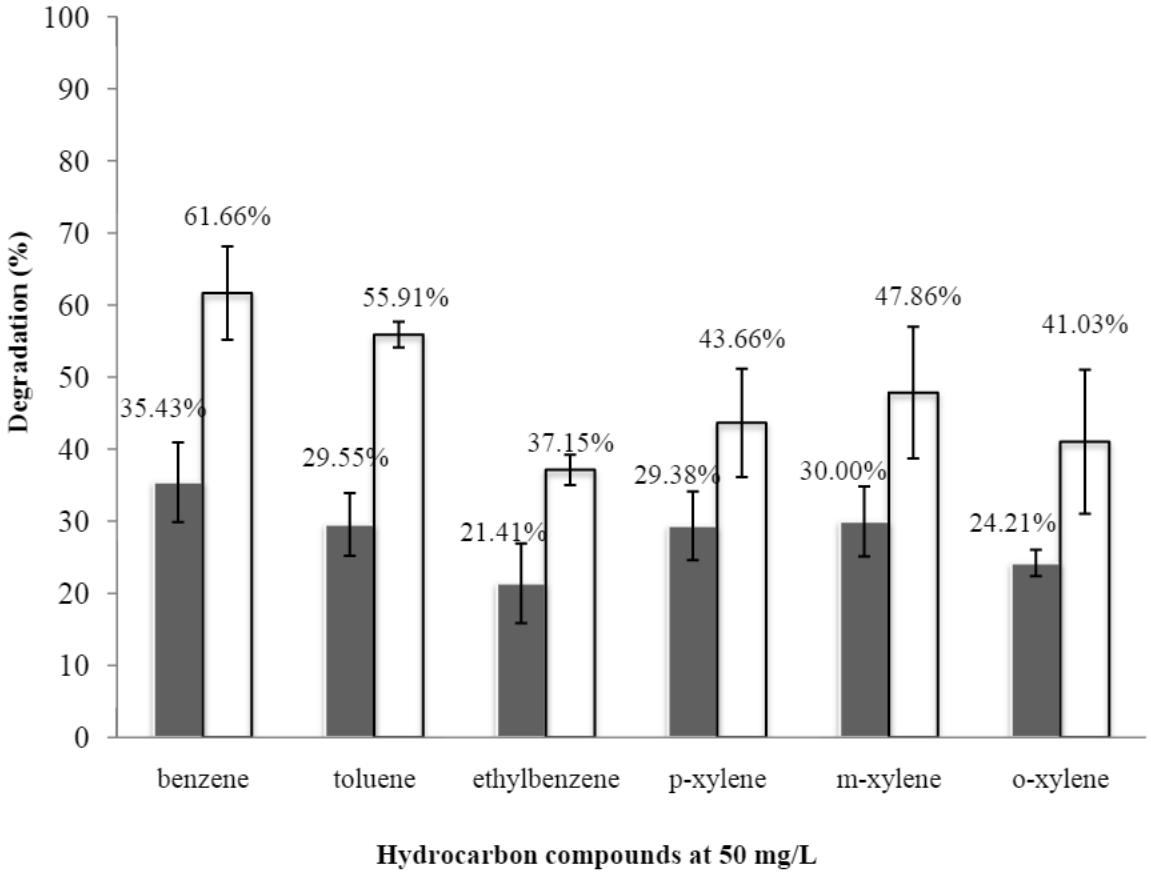

Fig. 3: BTEX degradation by CC after 24 (■) and 48 (□) hours of incubation. The biodegradation was performed at room temperature and agitated at 150 rpm for 48 hours with 5 % v/v CC treated with 50 mg/L benzene, toluene, ethylbenzene and xylene. Values are mean ± s.e.

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