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Research Article

Simultaneous Degradation of Trichloroethylene and Toluene by *Burkholderia cepacia* G4 and the Effect of Biotransformation on Bacterial Density

The effect of biomass density, effect of different ratios of trichloroethylene (TCE) and toluene on biomass, and strategies to cope with problems using acclimated *Burkholderia cepacia* G4 were investigated. Complete degradation of TCE was achieved when *B. cepacia* G4 was exposed to 0.5 mg/L TCE and 10 mg/L toluene after starvation of 100 h. Overall results of this study show that degradation rates of toluene decreased as TCE concentration increased. On the contrary, degradation rates of TCE increased up to 10 mg/L TCE and then decreased. Transformation capacity of *B. cepacia* G4 increased as toluene concentration increased but transformation yield decreased showing that excessive substrate supply may not help to improve specific performance parameters. Results of these experiments bear that instead of simultaneous injection of TCE and substrate, the supply of substrate in pulses can increase the efficiency of *B. cepacia* G4 or similar bacterial strains.

Keywords: Biological mineralization; Biomass density; Co-metabolism; Microorganisms; Optical density

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1 Introduction

Trichloroethylene (TCE) and toluene are one of the most frequently occurring organic pollutants in ground water and in some cases in domestic water supplies [1, 2]. Recent studies have shown strong relationship of TCE towards several types of cancers [3]. It is a priority pollutant in EPA's top twenty most dangerous pollutants list. TCE is biologically persistent to degradation by facultative microorganisms. However, some microorganisms produce non-specific enzymes, e.g., methane mono-oxygenase, toluene-2-mono-oxygenase. These non-specific enzymes can degrade TCE to simpler harmless end products through a biological oxidation process known as co-metabolism [4]. There is always a strong possibility that where TCE and toluene including compounds like benzene, toluene, ethyl benzene, and xylenes are found together in environment near industrial dumping sites [2]. In the situations where two or more pollutants are found, one of them can be used as supporting or primary substrate to degrade other using non-specific enzymes. For example, *Burkholderia cepacia* G4 degrade toluene and expresses toluene-2-mono-oxygenase on toluene exposure. Toluene-2-mono-oxygenase can degrade TCE via co-metabolism. Co-metabolism is an environmentally safe process through which TCE is mineralized to simple harmless end products (e.g., CO_2) unlike anaerobic biological dechlorination, which leaves toxic byproducts of TCE [3]. Biological mineralization of TCE through co-metabolism gives no benefits to microorganisms [5]. On the other hand, TCE and its transformation byproducts are toxic to microorganisms on short or long time exposure. Toxicity of TCE (exposed to degradation microorganisms may result in the reduction of bacterial cellular growth, viability, respiratory activity, and enzyme inactivation [6–11].

To solve toxicity related problems in co-metabolism many approaches have been reported in literature. Use of auxiliary or supportive non-aromatic substrates, employing the toxicity resistant bacterial strains and encapsulation are few examples [12, 13]. The use of auxiliary or supportive non-aromatic substrates has been reported in several studies, primarily to maintain viable active cells [12]. The induction of non-aromatic substrates resulted in depletion of oxygen in the subjected environment and competition between aromatic (e.g., toluene) and non-aromatic substrates (e.g., fructose, etc.). Using pure bacterial species (resistant to the toxicity of TCE and aromatic substrate) have shown better results [10]. *B. cepacia* G4 is one of the most extensively studied microorganisms, which produces toluene 2-mono-oxygenase on toluene induction and is resistant to TCE toxicity even on higher concentrations [8, 13, 14]. However, *B. cepacia* G4 may become sensitive to TCE degradation-dependent stress in conditions having limited growth substrate or energy [6]. There is still a debate going on this topic that under which conditions *B. cepacia* G4 may perform to its capacity. For example Mars et al. [6] used fed batch culture of *B. cepacia* G4 under non-growing conditions to assess TCE mediated toxicity while Yeager et al. [7] systematically examined the cytotoxicity related to TCE exposure with and without TCE. Using high toluene concentration (to avoid TCE mediated toxicity) is not only toxic to microorganisms but is also regulated by environmental agencies. On the other hand giving much low toluene may result in poor performance of *B. cepacia* G4. This means that behavior of acclimated *B. cepacia* G4 under growing conditions with mild to high TCE and toluene exposures still need more evaluation. The purpose of this study is to examine the behavior of acclimated *B. cepacia* G4

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Abbreviation: TCE, trichloroethylene

under growing conditions subjected to different combinations of TCE and toluene availability. This study suggests the optimal TCE/toluene and biomass combination(s) along with other strategies to improve efficiency of co-metabolic TCE removal in bioaugmentation and bioreactors.

2 Materials and methods

2.1 Culture growth and acclimation conditions

B. cepacia G4 (ATCC 53617) was purchased from the American type culture collection. To get high density biomass, the microorganisms were grown on autoclaved nutrient broth solution at 28°C overnight. The nutrient broth solution containing high biomass (optical density at 540 nm) was centrifuged and rinsed with sterile phosphate buffer saline for 5–10 min. Microorganisms were then cultured in phosphate buffered medium [15]. The composition of phosphate buffer saline, phosphate buffer medium and trace metal solution is given in Tab. 1 [15]. During the routine culture growth and acclimation, toluene was fed as sole energy and carbon source. The pH of the medium was 7.2 and the temperature was maintained at 28°C, kept in shaking incubator at 150 rpm. Pure oxygen was bubbled every second day to maintain super saturation condition, i.e., >30 mg/L. Acclimation and growth on toluene was carried out for approximately three months [16].

2.2 Cell density and protein assay

To determine the concentration of biomass, optical density at 540 nm (OD_{540}) in the solution was measured using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) [16]. Colorimetric method was employed to calculate proteins. The bovine serum albumin was used for protein assay. SMART™ BCA Protein Assay Kit (iNTRON Biotechnology) was used for this purpose, reading at 562 nm (UV-1601) [16].

2.3 Analytical methods

For TCE and toluene analysis, a gas chromatograph (Varian GC3400CX, USA) equipped with a flame ionization detector and a capillary column (DB-5, 0.53 mm, 30 m, J&W Scientific) was used. The injector and detector temperature were 200 and 230°C, respectively. The column temperature was maintained at 40°C for 3 min, and increased by 8°C/min until it reached 120°C [16]. The detection limit of the equipment used for both TCE and toluene is 1 μg/L. The concentration in liquid phase was calculated by Henry's law dimensionless constant (H_c) at 28°C. H_c for TCE and toluene was 0.445 and 0.298, respectively [15]. The TCE and toluene concentrations were calculated by the ratio of the chromatogram peak area.

For low range of TCE (0.1–1 mg/L) and toluene (10 mg/L), an aliquot of 100 μL was taken from head space of serum bottle with the help of gas tight syringe (Hamilton), and injected in GC. But for high range of TCE and toluene, the aliquot volume was taken at the rate of 10 μL and final values were multiplied by a factor of 10.99.8% pure toluene and 99.5% pure TCE (Sigma–Aldrich) were used for all experiments. Nutrient broth powder (Difco) was used for growth culture dissolved in deionized water (Millipore) and autoclaved.

2.4 General experimental conditions and design

All experiments were performed in batch mode and growing cells under aerobic conditions. Brown serum bottles (120 mL) were used to avoid photo catalysis of toluene and TCE. Temperature was maintained at 28°C and pH 7.2. Each reactor vial contained 50 mg/L sterilized mineral solution. Pure oxygen was purged into vials mineral solution for 2–3 min using sterile needle to get oxygen saturation condition. The pressure inside the serum bottle was maintained at atmospheric level. Teflon lined silicon crimp cap was used to avoid leakage and TCE adsorption to silicon. In all experiments, serum bottles were kept inverted in shaking incubator at 150 rpm to avoid losses due to leakage. All media and pans were autoclaved up to 120°C for 30 min prior to use. All the procedures were performed in clean bench to avoid any growth of microorganisms other than *B. cepacia* G4 [16].

Two ranges of TCE, i.e., 0.1–1 and 5–20 mg/L were investigated for five concentrations of toluene, i.e. 3, 5, 10, 20, and 50 mg/L. Initial biomass injected was about 120 mg/L (optical density of about 0.1 at 540 nm). Cells were injected into the serum bottle after achieving required concentration of TCE and toluene. OD_{540} at the rate of 1 was used for higher concentrations of TCE. All the experiments were repeated three to five times to get stable results along with control samples. The results of control experiments are not shown in figures to avoid complexity. A sketch of the experimental plan is described in Tab. 2.

3 Results and discussion

3.1 Effect of different toluene concentrations on TCE (low range) degradation and cell density

Degradation of TCE at low range (0.1, 0.3, and 1 mg/L) was evaluated with OD_{540} 0.1. Each TCE concentration at this range was subjected to degradation with four different concentrations of toluene (i.e., 3, 5, 10, and 20 mg/L). Figure 1a and b shows the degradation of toluene

Table 1. Composition of phosphate buffer saline, phosphate buffer medium, and trace metal solution

Phosphate buffer saline						
Salt	NaCl		Na_2HPO_4			$Na_2H_2PO_4$
Concentration (mg/L)	8.1		2.302			0.194
Phosphate buffer medium						
Salt	$(NH_4)_2SO_4$	K_2HPO_4	KH_2PO_4	$MgSO_4 \cdot H_2O$	$CaCl_2 \cdot 2\,H_2O$	$FeSO_4 \cdot 7\,H_2O$
Concentration (mg/L)	1.234	0.854	0.694	0.460	0.176	0.001
Trace metal solution						
Salt	$ZnSO_4 \cdot 7\,H_2O$	H_3BO_3	$CuCl_2 \cdot 2\,H_2O$	$CoCl_2 \cdot 6\,H_2O$	$MnCl_2 \cdot 4\,H_2O$	$NiCl_2 \cdot 6\,H_2O$	$Na_2MoO_4 \cdot 2\,H_2O$
Concentration (mg/L)	20	60	2	40	6	4	6

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Table 2. Layout of experimental design

No.	Toluene (mg/L)	TCE (mg/L)	OD_{540} (nm)
1	3, 5, 10, 20	0.1, 0.3, 1	0.1
2	10	5, 10, 20	0.1
3	50	5, 10, 20	0.1
4	10	0.5	0.1 and 1

(3, 5, 10, and 20 mg/L with TCE 0.1 mg/L while Fig. 1c shows the trends of *B. cepacia* G4 OD_{540} variations for TCE and toluene transformations. As toluene concentration increased, degradation rates of toluene also increased (Fig. 1a and Tab. 3). Similar trends were found in case of TCE degradation rates and extent of TCE removal increased as toluene supply increased (Fig. 1b). Maximum TCE (about 95%) was degraded by 20 mg/L toluene, which shows that proper supply of inducer/growth or energy substrate has the prime importance in co-metabolism. On the other hand, OD_{540} started decreasing in 3 and 5 mg/L of toluene from the start of experiment (Fig. 1c). Biomass initially increased slightly (0.114–0.13) for 10 mg/L toluene and then started decreasing after toluene was exhausted (Fig. 1c). The case was different with 20 mg/L toluene in which the OD_{540} increased slightly (from 0.11 to 0.135) but did not decrease much to the end of batch test. As 0.1 mg/L TCE is not toxic to acclimatized *B. cepacia* G4, the depletion of biomass under this condition was perhaps due to the shortage of growth substrate. Apparently, toluene at the rate of 3 and 5 mg/L was not sufficient to maintain the cell density. The results were better for higher toluene supply increase, i.e., up to 20 mg/L. Similar results have been reported in case of *Pseudomonas putida* F1 [16].

Figure 2a and b shows the optical density trends for TCE concentration of 0.3 and 1 mg/L, respectively, with the same range of toluene as above. The degradation trends of TCE and toluene were similar as observed in Fig. 1a and b (data not shown). The removal efficiency of TCE (data not shown) decreased as compared with the previous case (i.e., 0.1 mg/L TCE); however, the behavior of *B. cepacia* G4 biomass for 0.3 and 1 mg/L of TCE was similar as was observed at 0.1 mg/L TCE, except for 20 mg/L toluene. Instead of steady increase, there was steady decrease in OD_{540}, which could be due to slight increase in maintenance energy to cope with damage occurred by TCE (1 mg/L). It has been reported that as TCE concentration increases, removal efficiency decreases at same concentration of substrate [15, 17]. This trend has been related to decrease in NADH supply or increase in maintenance energy demand [9]. Hence, for the sustainable removal of TCE through co-metabolism; sufficient continuous supply of substrate is required to maintain the process efficiency and the biocatalyst (i.e., biomass in this case) at critical working level.

Table 3. Comparison of TCE and toluene degradation rates for 0.1 mg/L TCE at various concentrations of toluene

Toluene concentration (mg/L)	TCE rate (μg TCE/mg protein) per hour	Toluene rate (μg toluene/mg protein) per hour
3	0.089	34
5	0.098	53
10	0.114	69
20	0.237	147

3.2 Effects of different toluene concentrations on TCE (high range) degradation and cell density

The effect of the high TCE concentration and low toluene supply (10 mg/L) on biomass density along with the degradation of TCE and toluene was investigated and the results are presented in Fig. 3. *B. cepacia* G4 at the rate of $OD_{540} = 0.1$ were introduced at different initial TCE concentrations of 5, 10, and 20 mg/L.

As shown in Fig. 3, toluene degradation rates were reduced significantly as compared to those at low TCE concentrations (Figs. 1 and 2). No TCE degradation occurred at 20 mg/L but a slight degradation was observed at 5 and 10 mg/L TCE. On the other hand, OD_{540} decreased rapidly in the first 20 h for TCE concentrations of 10 and 20 mg/L. It is important to note that toluene degraded in about 25–30 h in all cases. During the same time span, OD_{540} decreased at 10 and 20 mg/L of TCE but it remained almost stable for TCE 5 mg/L (Fig. 3c). These results show that TCE $>$ 5 mg/L is more toxic to *B. cepacia* G4. It seems that, due to the less supply of toluene, the repair/growth mechanism was not as effective as have been reported [7, 8].

To re-assess the vitality of substrate availability, same concentrations of TCE were degraded with 50 mg/L toluene at same experimental conditions as above (Fig. 4). Unlike previous results (Fig. 3), TCE degradation was observed at all concentrations with an increased OD_{540}. Hence, the availability of high substrate can be considered essential at high TCE concentrations to maintain the biomass at critical level along with the TCE removal. Comparison of some key parameters (TCE degradation rate, toluene degradation rate, transformation yield, transformation capacity, and overall efficiency) is presented in Tab. 4 for both toluene concentrations (10 and 50 mg/L). It can be noted that the TCE degradation rates decreased significantly for 10 mg/L toluene but were stable for 50 mg/L toluene.

It is interesting to note that all parameters have higher values for 50 mg/L toluene compared with 10 mg/L toluene, except for the transformation yield (T_y). T_y is a specific efficiency term to show how

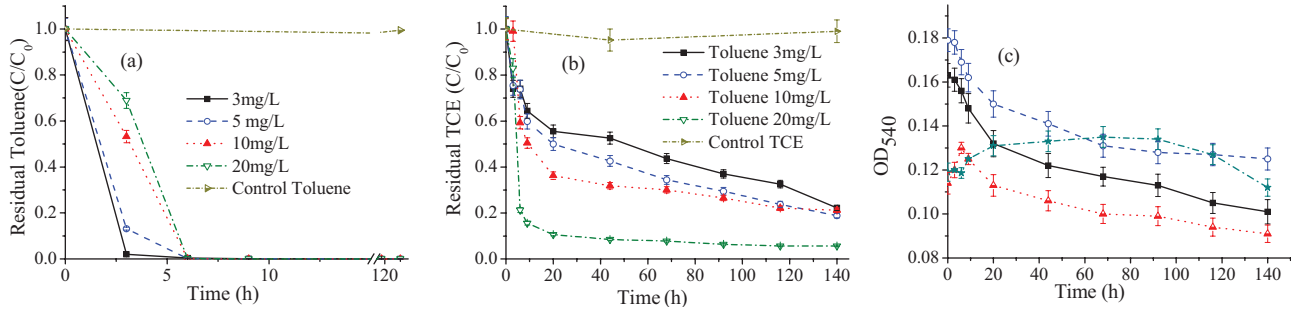

Figure 1. Simultaneous degradation of (a) toluene, (b) TCE (0.1 mg/L), and (c) variation in OD_{540}.

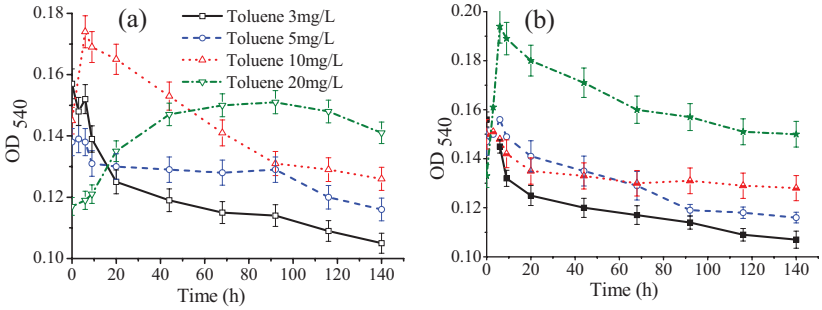

Figure 2. Variation in OD_{540} at various concentrations of toluene and (a) 0.3 mg/L TCE, (b) 1 mg/L TCE.

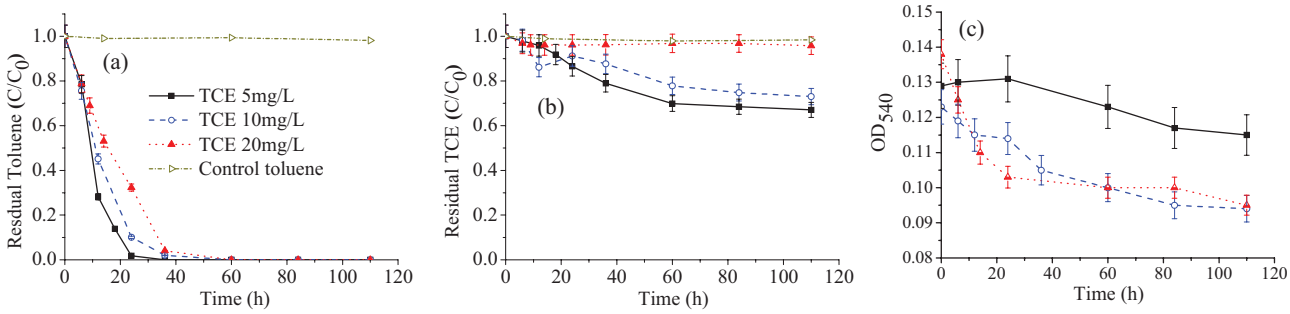

Figure 3. Simultaneous degradation of (a) toluene (10 mg/L) and (b) TCE, and (c) response of *B. cepacia* G4.

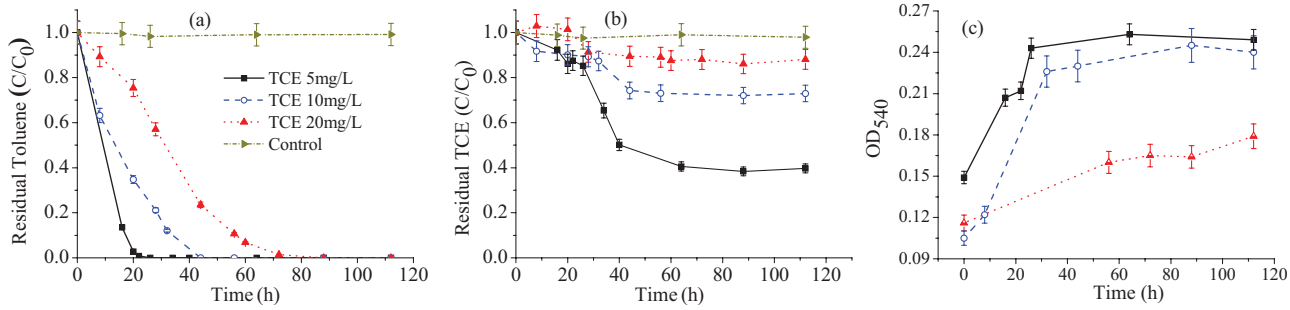

Figure 4. Simultaneous degradation of (a) toluene (50 mg/L) and (b) TCE, and (c) response of *B. cepacia*.

efficient a given substrate is used to transform TCE. Experiments with high substrate injection (i.e., 50 mg/L toluene) showed two distinct different results than those with low substrates. The first one was that *B. cepacia* G4 grown to double OD_{540} (Fig. 4c) and secondly T_y was less than those with low substrates. This result shows that *B. cepacia* G4 increased its population and used lesser amount of available substrates for TCE transformations. This behavior may be due to a phenomenon known as chemotaxis [18, 19]. Chemotaxis is a process in which microbes move towards substrate or optimal chemical concentrations and away from life hazards. This means that giving the high substrate may not solve the problems of toxicity and cell decay but may also disturb the very process of co-metabolism in parallel.

3.3 Effects of delayed injection of toluene on TCE degradation and OD_{540}

The supply of higher amount of substrates may not be a good idea to improve the overall process stability of co-metabolism, as discussed earlier (Figs. 3 and 4). Another experiment was conducted to evaluate

Table 4. Comparison of various parameters for 10 and 50 mg/L toluene with high TCE concentrations

	TCE rates (μg TCE/mg protein/h)		Toluene rates (μg toluene/mg protein/h)		T_c (mg TCE/mg protein)		T_y (mg TCE/mg toluene)		Overall removal efficiency (%)	
TCE (mg/L)	Tol[a] 10 mg/L	Tol 50 mg/L	Tol 10 mg/L	Tol 50 mg/L	Tol 10 mg/L	Tol 50 mg/L	Tol 10 mg/L	Tol 50 mg/L	Tol 10 mg/L	Tol 50 mg/L
5	1.395	3.330	24	55.939	0.064	0.080	0.157	0.069	32.93	60.29
10	3.195	2.232	20	31.178	0.116	0.064	0.237	0.067	26.96	27.07
20	1.541	3.693	10.36	33.47	0.030	0.103	0.078	0.066	4.12	12

[a] Tol, toluene.

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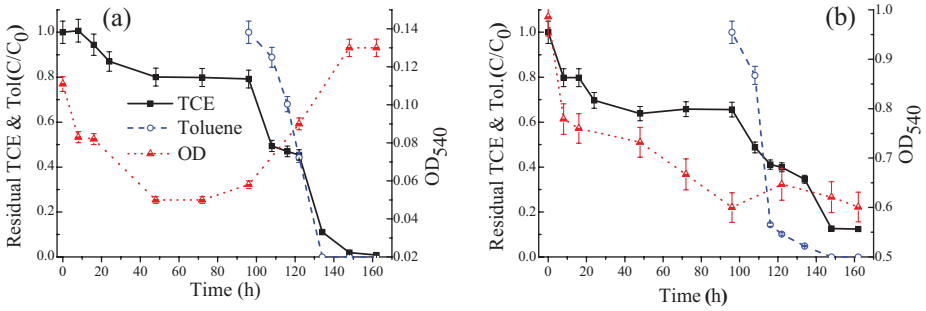

Figure 5. Response of *B. cepacia* G4 to TCE (0.5 mg/L) and (a) delayed injection of toluene (10 mg/L) for OD = 0.1 and (b) delayed injection of toluene (10 mg/L) for OD = 1.

the behavior of *B. cepacia* G4 with delayed injectioning of toluene at 100 h (Fig. 5). Two biomass concentrations were used ($OD_{540} = 0.1$ and 1). TCE was fed at the rate of 0.5 mg/L in both cases. Toluene (10 mg/L) was fed once the TCE degradation came to a halt (after 100 h from the launch of the experiment). *B. cepacia* G4 was grown on toluene for only three months.

Figure 5a shows the degradation of 0.5 mg/L TCE at $OD_{540} = 0.1$. Initially about 20% of TCE was degraded by resting cells. After about 40 h, no degradation was observed and TCE concentration also remained constant. In the same time, OD_{540} decreased sharply to about 30%. This was apparently due to the lack of growth substrate because TCE at 0.5 mg/L is not toxic to cells, as observed earlier (Figs. 2 and 3). After toluene injection (at 100 h), simultaneous degradation of TCE and toluene was observed at relatively faster rates than that was observed when both were fed simultaneously at 0 h. Both TCE and toluene were completely degraded within 35–60 h of toluene injection. This is different from our previous results, in which 0.1 and 0.5 mg/L TCE was not completely degraded at 10 mg/L toluene (only about 95 and 50% degradation was observed, respectively, for 0.1 and 0.5 mg/L TCE, Fig. 1), when TCE and toluene was injected at the same time under same conditions. Similar trends have been reported for *P. putida* F1 with toluene and TCE degradation [20, 21].

For $OD_{540} = 1$ (Fig. 5b), higher TCE degradation (about 35%) was observed when compared with $OD_{540} = 0.1$ (20%). After the injection of toluene, a fast removal of TCE and toluene was observed but with a little recovery of cell density. Despite of high cell biomass and same toluene injection, 0.5 mg/L of TCE was not completely degraded at $OD_{540} = 1$ (Fig. 5b). Results of these experiments bear that instead of simultaneous injection of TCE and substrate, the supply of substrate in pulses can increase the efficiency of *B. cepacia* G4 or similar bacterial strains. To avoid the loss of biocatalyst, the delayed injectioning of toluene may be done earlier, e.g., at 60 h (Fig. 5a, where the TCE concentration after the initial degradation remains constant from 60 to 100 h), depending upon the initial degradation of TCE by starving cells. The behavior of high biomass ($OD_{540} = 1.0$) supports the assumption that cells tend to use the available substrate to repair the damage done by TCE and/or to fulfill their energy demands and cell growth.

3.4 Behavior of kinetic parameters with $OD_{540} = 0.1$ and toluene 10 mg/L

Figure 6a and b shows the TCE/toluene degradation rates and transformation capacity (T_c)/yields (T_y), respectively, for $OD_{540} = 0.1$ and toluene supply of 10 mg/L. T_c and T_y are two different concepts that are used to describe how effective and efficient is the co-metabolic degradation of TCE [9]. T_c represents the quantity of TCE degraded per unit mass of microorganisms and is given by mass of TCE (metabolite) per unit mass of microorganisms. T_y is the maximum mass of TCE degraded per unit mass of substrate used to grow microorganism [5]. TCE degradation rates increased with increased TCE concentrations but toluene degradation rates decreased. This is in accordance with the literature [7, 8]. It has been stated that TCE and its byproducts produced during co-metabolism are harmful for cell activities. Hence toluene degradation rate decreases with increase in TCE [15].

In this study, T_y and T_c increased simultaneously up to 10 mg/L TCE but decreased afterwards. This indicates that TCE concentration beyond 10 mg/L is toxic to suspended *B. cepacia* G4. Similar trends have been reported for *P. putida* F1 using phenol as substrate [17, 22]. A range from 0.0021 to 0.0025 mg TCE/mg toluene has been reported in the literature for mixed cultures chemostates. For phenol, as a co-substrate, a range from 0.052 to 0.222 mg TCE/mg phenol has

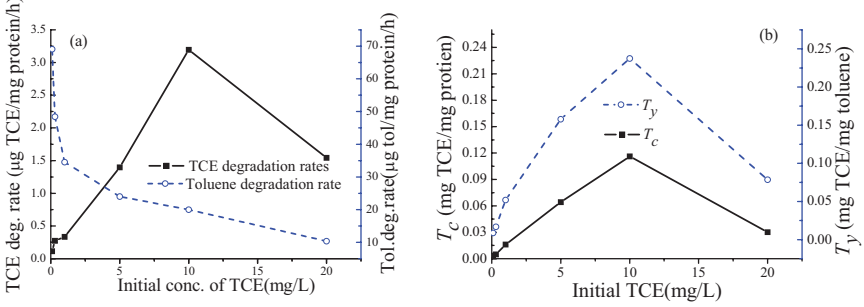

Figure 6. Trends of (a) TCE and toluene degradation rates, (b) transformation capacity (T_c) and transformation yield (T_y).

been reported using mixed cultures and phenol as a substrate [23]. A range of T_y (0.002–0.106 μmol of TCE/μmol of substrate) values was reported by Tejasen [22].

4 Concluding remarks

The purpose of this study was to examine the behavior of acclimated *B. cepacia* G4 with different combinations of TCE and toluene aiming at the sustainable and complete removal of TCE. For this purpose, *B. cepacia* G4 was acclimated to toluene for 3 months to enhance its resistivity against TCE/toluene toxicity. At low toluene supply (3, 5, 10, and 20 mg/L), degradation of TCE decreased along with OD_{540}, as initial TCE concentration increased from 0.1 to 1 mg/L at respective toluene concentration. At 10 mg/L of toluene, TCE removal as well as cell's biomass was greatly suppressed at 10 and 20 mg/L TCE. Despite an incomplete TCE removal at 50 mg/L toluene, activity of biomass and degradation rates of TCE enhanced considerably (percentage removal of TCE increased by about 50%) than at 10 mg/L toluene. The low specific removal of TCE at 50 mg/L, however, required additional experimental modifications (e.g., delayed injection of toluene in this study) in addition to the high supply of toluene that can achieve the complete removal of TCE. Hence, toluene supply (10 mg/L) after about 100 h of TCE exposure to *B. cepacia* G4 resulted complete degradation of TCE at 0.5 mg/L as well as rehabilitation of OD_{540} to its initial level. It is, thus, recommended that supply of substrate in pulse or cycles may not only increase TCE degradation efficiency but may also help to maintain proper *B. cepacia* G4 on such a level that all the biomass is utilized in co-metabolic activity. Both T_y and T_c increased simultaneously up to 10 mg/L TCE but decreased afterwards indicating that beyond this value, TCE is toxic to suspended *B. cepacia* G4. Finally, this study reveals that high concentration of acclimated biomass may not degrade even smaller amount of TCE without proper TCE/toluene, cell biomass ratios and substrate supply plan.

The authors have declared no conflict of interest.

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