ARTICLE

Degradation of chlorotoluenes by *Comamonas testosterone* KT5

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Abstract The isolated *Comamonas testosterone* KT5 utilized a broad range of toluene and chlorotoluenes, including 2-chlorotoluene, 3-chlorotoluene (3CT), 4-chlorotoluene, 2,3-dichlorotoluene, 2,4-dichlorotoluene, 2,6-dichlorotoluene and 3,4-dichlorotoluene (34DCT) as sources of carbon and energy. The strain was characterized its dissipation capability toward these compounds in both liquid culture and contaminated soil. In liquid cultures, KT5 utilized more than 90% of toluene, 3CT and 34DCT within 60 h at the initial concentration of 2 mM. Moreover, the strain showed a mineralization capacity of mixtures of toluene and chlorinated toluenes. Inoculation with the toluenes-degrading bacterial strain significantly enhanced degradation rates in soil. The dissipation rates of toluene, 3CT and 34DCT in non-sterile soil inoculated with bacteria were 97.8, 93.5 and 68.9% after 30 days, respectively. The biodegradation of toluene and chlorosubstituted toluenes in KT5 was occurred through the upper pathway to form benzoates and then ring fission via *ortho*-cleavage pathway. In addition, *C. testosterone* KT5 showed the mineralization capacities of benzoate and chlorinated benzoates with the rates comparably higher than the rates of toluenes. The multiple and efficient toluene degradation properties make this isolate a good candidate for bioremediation of environments contaminated with chlorosubstituted toluenes and benzoates.

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Introduction

Chlorotoluenes are used in production of agro-chemicals, flame retardants, dyes, pesticides, varnish and pigments, textile additives, pharmaceuticals, adhesives, polymers and resins, drain cleaners, and other products [1–4]. Because of these applications, chlorotoluenes were produced in large amounts [3, 4]. The compounds are moderately toxic chemicals [5, 6]. They have been widely detected in environments, including in soil, groundwater and surface waters [7–12]. Moreover, chlorotoluenes are used as cleaning agents and solvents leading to detect in room air [13] and convenience foods [14].

Because of their toxicity, the removal of these substrates contaminated in environments is necessary. One effective and environmental friendly way to remove toluenes from environments is through biodegradation. The biodegradation of chlorotoluenes, including mono-, di- and trichlorotoluenes has been investigated [3, 15–25]. Although the biodegradation of toluene and several chlorotoluenes is well documented in liquid media, there is no publication describing the biodegradation of a mixture of mono- and dichlorotoluenes as a sole carbon source. Moreover, the bioaugmentation of bacteria in soils has not been investigated.

In the biodegradation pathways of chloroaromatics, peripheral enzyme reactions are usually initiated to activate the aromatic ring and a special chlorocatechol pathway [26]. Degradation can be initiated by dioxygenation to form chloromethyl substituted catechols called lower

degradation pathway. The degradation in the lower pathway of toluene and its chlorine derivatives usually resulted in complete mineralization [16, 27–29]. In the second pathway (upper pathway), the reaction is initiated via oxidation of the methyl, which occurred via monooxygenation of the side chain of chlorotoluenes to transform into the respective benzoates and catechols and harboring a chlorocatechol pathway for degradation of chlorocatechols [15]. Nevertheless, due to the restricted substrate specificity of oxygenase, chlorosubstituted toluenes cannot be completely mineralized [15, 30, 31]. The engineering using a monooxygenolytic pathway was even failed to complete mineralize toluenes [14]. Thus, the isolation of microorganisms which can completely mineralize toluene and its chlorine derivatives as well as analysis of the toluenes-degrading enzymes should be done. In this study, the characterization of the biodegradation in liquid, soil and enzymes involving the degradation pathway toward chlorosubstituted toluenes by *C. testosterone* KT5 is investigated.

Materials and methods

Enrichment, isolation and identification of the bacterial stain

Soil, mud, river sediment, sewage sludge samples in several hazardous waste sites and industrial effluents in South of Viet Nam were collected and mixed thoroughly. Five grams of the sample was transferred into a flask containing 200 mL mineral medium (MM). A mixture of 2CT, 3CT, 4CT, 23DCT, 24DCT, 25DCT, 26DCT, 34DCT and 35DCT (0.1 mM each) was added into the solution. Chlorotoluenes were dissolved in absolute ethanol as stock solutions prior to use. The components of MM medium were described by Dobslaw and Engesser [3] with modification. MM medium contained (in grams per liter) Na_2HPO_4, 2.79; KH_2PO_4, 1.00; $(NH_4)_2SO_4$, 1.00; $MgSO_4 \cdot H_2O$, 0.20 and 1.00 mL trace mineral solution. The trace mineral solution consisted (in grams per liter) of H_3BO_3, 0.30; $CoCl_2 \cdot 6H_2O$, 0.20; $ZnSO_4 \cdot 7H_2O$, 0.10; $Na_2MoO_4 \cdot 2H_2O$, 0.03; $MnCl_2 \cdot 4H_2O$, 0.03; $NiCl_2 \cdot 6H_2O$, 0.02; $CuCl_2 \cdot 2H_2O$, 0.01. pH of the MM was adjusted at 7.0 ± 0.1. The medium was autoclaved at 121 °C for 15 min. The enrichment culture was incubated at room temperature (around 30 °C), 150 rpm for 1 month. Mixtures of chlorotoluenes were supplemented every week. Chemicals were purchased from Sigma–Aldrich or Merck.

Sample solution was the diluted and spread on solid MM supplemented with the mixture of chlorotoluenes. Single colonies were stroke on new plates. Genomic DNA samples were extracted using an InstaGene[tm] Matrix (Bio-Rad Laboratories, Hercules, CA, USA). The 16S rRNA gene fragment was PCR amplified using the universal primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) at 95 °C for 2 min and then 35 cycles of 95, 55 and 72 °C for 1 min each, followed by 10 min at 72 °C. The purification of amplification products was carried out using a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing was performed using a PRISM BigDye Terminator v3.1 cycle sequencing kit. Related sequences were determined using BLAST and obtained from the GenBank database (National Center for Biotechnology Information).

Biodegradation in liquid culture

The degradation experiments by freely suspended cells were carried out aerobically at room temperature and 150 rpm. The culture was done at the early beginning of the exponential phase with optical density of 0.1 at 600 nm. The utilization by bacteria of a single compound of toluenes and benzoates was examined at an initial concentration of 2 mM each. For determination of the effects of cosubstrates on biodegradation rates, individual cosubstrates of glucose, succinate, yeast extract and sodium nitrate (0.1%) were supplemented. The utilization of a mixture of toluene and chlorinated toluenes was taken at an initial concentration of 0.5 mM of each compound.

Biodegradation of individual chlorotoluenes in soil

The soil samples were taken from a depth of 10–50 cm in Dong Thap University campus, where the chlorotoluenes had never contaminated. The soil was then air-dried at room temperature, mixed thoroughly, and sieved through 2 mm to remove large debris and ensure homogeneous mixing. The soil sample contained 43.6% sand, 34.7% silt, 19.0% clay. Other physicochemical properties included 21.4 g kg^{-1} dry weight of organic matter, 1.27 g kg^{-1} dry weight of total nitrogen, 1.13 g kg^{-1} dry weight of total phosphorus, 1.56 g cm^{-3} of bulk density and had a pH value of 6.6.

Soil was spiked with 1.0 mM kg^{-1} of individual toluenes. The soil was inoculated with the cell suspension to give an initial population of 10^6 cells g^{-1} dry soil. Soil (200 g) was transferred to 500-mL glass jars covered with aluminum foil. The jars were shaken every 5-day period to enhance soil O_2 availability. The soil microcosms were incubated at room temperature in the dark condition during the experiment. Soil moisture was maintained at 25% of the water-holding capacity by sprinkling sterile water. Uninoculated controls were run in parallel. For determination of chlorotoluenes degraded in soil, 5 g soil was collected and then extracted with 15 mL volume of

methylene chloride (CH_2Cl_2) twice. The extract was concentrated and filtered with a 0.22-μm syringe filter. The mean recovery efficiency from the soil was 92.4% of toluene, 95.5% of 3CT and 93.2% of 34DCT.

For bacterial inoculum preparation for soil experiments, the bacterial suspension was cultured in 500-mL flasks containing 200 mL MM medium supplemented with 0.1% succinate for 12 h. The culture was then centrifuged for 5 min at 12,000 rpm. Bacterial cells were washed twice with phosphate buffer (50 mM, pH 7.0) and resuspended in 20 mL of MM medium.

Enzyme assays

Cells cultured in MM medium supplemented with 2 mM individual chemicals for 48 h were harvested by centrifugation for 5 min at 10,000 rpm. Cells were then washed twice and resuspended with 50 mM Tris–HCl buffer pH 7.5. The cells were disrupted by sonication for 5 min and centrifuged at 10,000 rpm for 10 min at 4 °C to remove cell debris. The supernatant was used for enzyme assays. Catechol 1,2-dioxygenase activity was assayed spectrophotometrically by measuring the formation of cis, cis-muconic acid of catechol at 260 nm [32]. Catechol 2,3-dioxygenase activity was assayed according to Urata et al. [33] by following the formation of 2-hydroxymuconic semialdehyde, the meta-cleavage product of catechol, at 375 nm.

Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase were assayed as the method described by Worsey and Williams [34]. The activities of these enzymes were measured by following the reduction rate of NAD^+ to produce NADH at 340 nm. For benzyl alcohol dehydrogenase, the reaction mixture containing 250 μM of tris(hydroxymethyl) aminomethane-hydrochloride buffer (pH 8.5), 10.0 μM of NAD^+, 10.0 μM of substrate. For benzaldehyde dehydrogenase, the reaction mixture including 250 μM of sodium glycinate buffer (pH 9.4), 10.0 μM of NAD^+, 1.5 μM of substrate. One unit of enzyme activity was defined as the amount of enzyme catalyzing to produce 1 μM of product in 1 min at 30 °C. Cell extract in a volume of 3.5 mL was used for both enzyme assays.

Analytical methods

HPLC Model 600E using spherisorb C18 5 UV, 4.5×250 mm column was applied for the determination of chemical remaining. The mobile phase was methanol at the flow rate of 0.5 mL min^{-1}. The Waters UV detector model 2487 was used at 254 nm. In addition, gas chromatograph–mass spectrometry (GC–MS) (Agilent, USA) analysis was employed for determination of chemical concentrations. Cell turbidity was determined at 600 nm using a DU800 spectrophotometer (Beckman Coulter Inc., Brea, CA, USA), and the exponential growth rate was determined based on Zeyer et al. [35]. All experiments were conducted at least three time replicates. The modified Lowry method [36] was used to determine the protein concentrations, using bovine serum albumen as the protein standard.

Statistical analysis

The data are shown as the mean $\pm$ one standard deviation. Significant differences among means were statistically analyzed using one-way ANOVA with Duncan's test (Statistical Package for Social Sciences (SPSS) program version 22.0).

Results

### Isolation and Identification of the bacterial strain

After separation and enrichment, several bacterial strains which could utilize toluene and chlorinated toluenes as sole carbon and energy sources were isolated. The strain with the highest degradation rate was named strain KT5. KT5 was an aerobic, Gram-negative bacterium and was well cultivatable at room temperature and pH around 7.0. The analysis of the 16S rRNA gene showed that KT5 exhibited a high sequence similarity to *C. testosterone* (>99.9% homology).

Biodegradation in liquid culture by freely suspended *C. testosterone* KT5

C. testosterone KT5 utilized a broad range of toluene, mono- and dichlorotoluenes (Figs. 1, 2). The degradation of these chemicals as sources of carbon and energy occurred at different rates. Toluene was degraded with highest rate, followed by 3CT and 34DCT. KT5 showed slowly to degrade 2CT. 2CT was completely degraded after 4 days of incubation at the initial concentration of 2.0 mM. However, the strain insignificantly degraded 25DCT and 35DCT (not over 15.2% after 4 days).
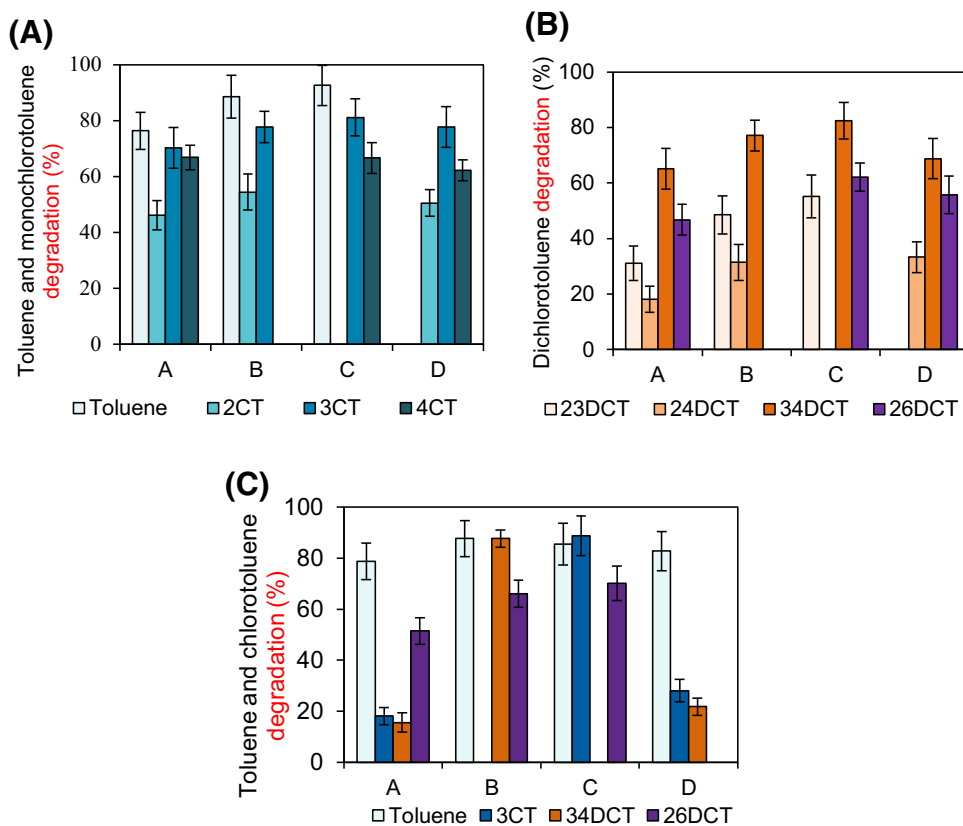
The determination of degradation rates in mixtures of 3 or 4 compounds of toluenes revealed the influences of one on the degradation of other compounds. The degradation rates of mono- and dichlorotoluenes in the mixture are presented in Fig. 2A, B. The presence of both 3CT and 34DCT in a mixture resulted in inhibition of degradation each other, while they did not affect the degradation of other substrates (Fig. 2C).
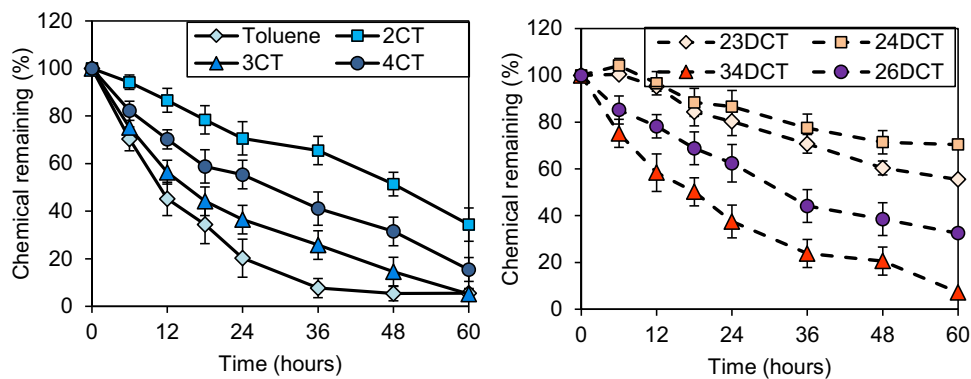
Fig. 1 Utilization curve of individual toluene and chlorinated toluenes (2 mM each) by batch cultures of strain KT5 as sources of carbon during cell growth in MM medium. The substrates included toluene, 2-chlorotoluene (2CT), 3-chlorotoluene (3CT) and 4-chlorotoluene (4CT), 2,3-dichlorotoluene (23DCT), 2,4-dichlorotoluene (24DCT), 3,4-dichlorotoluene (34DCT) and 2,6-dichlorotoluene (26DCT)

Fig. 2 Utilization of chemical mixtures (0.5 mM each) by batch cultures of strain KT5 as sources of carbon during cell growth after 60 h of incubation. Treatment A included 4 compounds, and B, C and D consisted of 3 compounds. The substrates included toluene, 2-chlorotoluene (2CT), 3-chlorotoluene (3CT), 4-chlorotoluene (4CT), 2,3-dichlorotoluene (23DCT), 2,4-dichlorotoluene (24DCT), 2,6-dichlorotoluene (26DCT) and 3,4-dichlorotoluene (34DCT)

Further investigation was carried out to determine the effect of cosubstrates as alternative sources of carbon/nitrogen on toluene and chlorinated toluenes biodegradation rates and growth of stain KT5. The addition of any cosubstrate stimulated the cell growth rates (Table 1). The presence of glucose in medium did not significantly affect the degradation rates, while succinate stimulated the degradation rates of any compound. For nitrogen sources, ammonium sulfate in MM medium served as a nitrogen source of KT5. The addition of nitrate as another nitrogen source did not significantly affect the biodegradation. By contrast, the degradation rates enhanced with the supplementation of yeast extract.

In addition, the determination of benzoate and chlorinated benzoates degradation presented that KT5 utilize from 62 to 98% of 2 mM individual benzoate, 2-chlorobenzoate (2CB), 3-chlorobenzoate (3CB) and 4-chlorobenzoate (4CB), 2,3-dichlorobenzoate (23DCB), 2,4-dichlorobenzoate (24DCB), 3,4-dichlorobenzoate (3,4DCB) and 2,6-dichlorobenzoate (26DCB) within 48 h

Table 1 Effects of cosubstrates on toluene and chlorotoluene utilization

Substrates	Cosubstrates	Degradation rates (%)*	Exponential growth rate (h^{-1})*
Toluene	None	79.7 ± 7.8^{defg}	0.055 ± 0.008^{bcd}
	Glucose	67.5 ± 3.3^{abc}	0.070 ± 0.011^{defg}
	Succinate	89.5 ± 5.5^{gh}	0.082 ± 0.012^{fg}
	Sodium nitrate	80.5 ± 6.1^{efg}	0.071 ± 0.008^{efg}
	Yeast extract	92.5 ± 4.1^{h}	0.085 ± 0.011^{g}
3-chlorotoluene	None	69.5 ± 7.1^{bcd}	0.046 ± 0.007^{ab}
	Glucose	66.5 ± 6.8^{abc}	0.070 ± 0.008^{defg}
	Succinate	84.5 ± 4.0^{fgh}	0.071 ± 0.010^{efg}
	Sodium nitrate	74.5 ± 5.5^{cde}	0.068 ± 0.009^{bcde}
	Yeast extract	85.5 ± 4.4^{fgh}	0.075 ± 0.011^{efg}
3,4-dichlorotoluene	None	62.5 ± 6.8^{ab}	0.038 ± 0.004^{a}
	Glucose	58.5 ± 6.5^{a}	0.062 ± 0.006^{bcd}
	Succinate	79.5 ± 6.1^{defg}	0.068 ± 0.006^{bcde}
	Sodium nitrate	70.5 ± 6.5^{bcd}	0.052 ± 0.004^{abc}
	Yeast extract	81.5 ± 3.8^{fg}	0.061 ± 0.005^{bcd}

Toluene and chlorotoluenes were added at 2 mM, and cosubstrates were added at 0.1%. Data were obtained after 24 h of incubation

* Different superscript letters indicate statistically significant differences ($p < 0.05$) among treatments within a column using the one-way ANOVA with Duncan's test in SPSS software
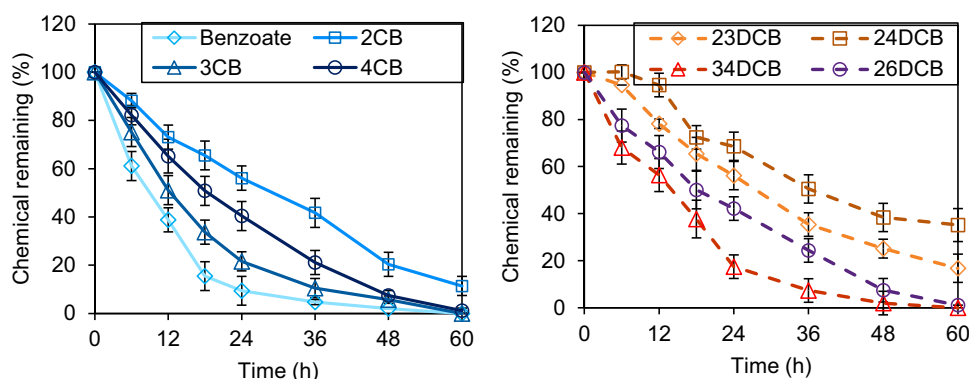
Fig. 3 Utilization curve of individual benzoate and chlorinated benzoates (2 mM each) by batch cultures of strain KT5 as sources of carbon during cell growth in MM medium. The substrates included benzoate, 2-chlorobenzoate (2CB), 3-chlorobenzoate (3CB) and 4-chlorobenzoate (4CB), 2,3-dichlorobenzoate (23DCB), 2,4-dichlorobenzoate (24DCB), 3,4-dichlorobenzoate (3,4DCB) and 2,6-dichlorobenzoate (26DCB)

(Fig. 3). In abiotic controls, toluene, chlorinated toluene, benzoate and chlorinated benzoates losses were negligible (data were not shown).

Bioremediation of toluene and chlorinated toluenes contaminated soil

The degradation of toluene compounds in soil is presented in Table 2. After 15 and 20 days, the dissipation rates in soil inoculated with KT5 were significantly higher than that in non-inoculated soil with or without sterilization. The reduction of toluene, 3CT and 34DCT in non-sterilized soils without bacterial suspension of KT5 was not over

26% after 30 days (Table 2). The inoculation of *C. testosterone* KT5 resulted in higher rates of chemical loss compared with the soil without any bacteria inoculated. The degradation rates of toluene, 3CT and 34DCT in non-sterile soil inoculated with bacteria were 97.8, 93.5 and 68.9% after 30 days, respectively, which were considerably higher than that in sterile soil.

Chlorotoluenes degradation pathway in *C. testosterone* KT5

The investigation of degradation pathway of toluene, 3CT and 34DCT in the isolate was conducted by determining

Table 2 Biodegradation of individual toluene and chlorinated toluenes in soil (1.0 mM each)

Treatments	Substrates	Toluene and chlorinated toluenes remaining in soil (%)*			
		15 days		30 days	
		Sterile soil	Non-sterile soil	Sterile soil	Non-sterile soil
Non-inoculation	Toluene	90.4 ± 2.4^{Bd}	82.2 ± 4.6^{ABd}	88.8 ± 3.3^{Bc}	77.7 ± 7.1^{Ac}
	3CT	91.2 ± 3.3^{Bd}	89.4 ± 5.3^{Bd}	90.3 ± 4.1^{Bc}	81.4 ± 2.3^{Ac}
	34DCT	93.3 ± 3.7^{Ad}	90.2 ± 4.5^{Ad}	91.1 ± 3.3^{Ac}	86.1 ± 5.5^{Ac}
Inoculation with *C. testosterone* KT5	Toluene	35.4 ± 4.1^{Da}	22.4 ± 4.7^{Ca}	12.6 ± 4.4^{Ba}	2.2 ± 1.1^{Aa}
	3CT	46.6 ± 4.4^{Db}	31.4 ± 4.5^{Cb}	17.6 ± 5.2^{Ba}	6.5 ± 2.3^{Aa}
	34DCT	68.4 ± 5.4^{Cc}	60.1 ± 5.6^{Cc}	42.4 ± 4.2^{Bb}	31.1 ± 5.5^{Ab}

* Different capital and small superscript letters indicate statistically significant differences ($p < 0.05$) among treatments within a line and a column, respectively

activities of the enzymes involved. The activities of enzymes extracted from cells grown on media containing toluenes were carried out in comparison with the cells grown on the medium with succinate as the sole carbon source. The enzyme activities were examined on proposed intermediates in the pathway for their degradation. These substrates are degraded by oxidation of the methyl substituent via the corresponding alcohols and aldehydes to benzoate and corresponding benzoates, which are then further ring fission. Tables 3 and 4 indicate that both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase were present during cell growth on various substrates. The activities of enzymes extracted from cells grown on any toluene were insignificantly different but higher than cells grown on succinate (Tables 3, 4).

Further determination of activities of enzymes responsible for the ring fission was also carried out. The activities of catechol 1,2-dioxygenase (*ortho*-cleavage pathway), and catechol 2,3-dioxygenase (*meta*-cleavage pathway) were examined from toluene and substituted toluenes grown cells. The activity of catechol 1,2-dioxygenase was noticeably induced, while catechol 2,3-dioxygenase activity was insignificant (Table 5). These results indicated that the *meta* degradation pathway was negligible. Moreover, the lower pathway intermediate products such as

3-methylcatechol produced from the degradation of toluenes, 3-chloro-5-methylcatechol and 5-chloro-3-methylcatechol produced from the degradation of 3CA [37] were not detected during the incubation. Enzymes responsible for the side chain oxidation activity and ring fission were also inducible in media containing succinate as the sole carbon source.

Discussion

The determination of the mixture of toluene and substituted toluene compounds transformed by microorganisms should be determined before the application of bioremediation. The contaminated environmental sites generally contained chlorosubstituted toluene mixtures [10, 14, 38]. In previous reports, no native and genetically engineered bacterial strains were clearly stated for mineralizing toluene and chlorinated toluenes in a mixture. 2CT-degrading *Rhodococcus* sp. OCT 10 could not degrade other substituted toluene compounds [3]. *Pseudomonas* sp. PS12 degraded toluene and some chlorotoluenes but did not degrade 2CT, 3CT, 25DCA and 26DCA [35]. *Ralstonia* sp. strain PS12 could utilize 24DCT, 25DCT with higher rates than 34DCT [28], which was different from the

Table 3 Relative specific activities of benzyl alcohol dehydrogenase in *C. testosterone* KT5 grown on various carbon sources

Growth substrates	Specific activities [Units.(mg protein)$^{-1}$] for substrates*		
	Benzyl alcohol	3-chlorobenzyl alcohol	3,4-dichlorobenzyl alcohol
Toluene	1.13 ± 0.21^{Bc}	0.82 ± 0.14^{ABbc}	0.71 ± 0.11^{Ab}
3CT	0.91 ± 0.15^{Bbc}	0.76 ± 0.11^{ABb}	0.65 ± 0.12^{Ab}
34DCT	0.82 ± 0.12^{Bb}	0.65 ± 0.10^{ABb}	0.60 ± 0.08^{Ab}
Succinate	0.23 ± 0.03^{Aa}	0.22 ± 0.06^{Aa}	0.20 ± 0.05^{Aa}

* Different capital and small superscript letters indicate statistically significant differences ($p < 0.05$) among treatments within a line and a column, respectively

Table 4 Relative specific activities of benzaldehyde dehydrogenase in *C. testosterone* KT5 grown on various carbon sources

Growth substrates	Specific activities [Units.(mg protein)$^{-1}$] for substrates[*]		
	Benzaldehyde	3-chloro tolualdehyde	3,4-dichloro tolualdehyde
Toluene	1.00 ± 0.11[Bc]	0.82 ± 0.14[ABb]	0.65 ± 0.11[Ab]
3CT	0.74 ± 0.12[Ab]	0.76 ± 0.11[Ab]	0.54 ± 0.08[Ab]
34DCT	0.71 ± 0.11[Bb]	0.65 ± 0.10[ABb]	0.52 ± 0.04[Ab]
Succinate	0.26 ± 0.05[Aa]	0.22 ± 0.06[Aa]	0.18 ± 0.02[Aa]

[*] Different capital and small superscript letters indicate statistically significant differences ($p < 0.05$) among treatments within a line and a column, respectively

Table 5 Relative specific activities of catechol-1,2-dioxygenase and catechol 2,3-dioxygenase in *C. testosterone* KT5 grown on various carbon sources

Growth substrates	Specific activities [Units.(mg protein)$^{-1}$] for substrates[*]	
	Catechol-1,2-dioxygenase	Catechol 2,3-dioxygenase
Toluene	1.7 ± 0.21[Bc]	0.01 ± 0.00[A]
3CT	1.2 ± 0.13[Bb]	0.02 ± 0.01[A]
34DCT	1.1 ± 0.30[b]	0
Benzoic acid	2.5 ± 0.31[Bd]	0.02 ± 0.01[A]
3-chlorobenzoate	2.1 ± 0.20[Bc]	0.01 ± 0.00[A]
3,4-dichlorobenzoate	1.8 ± 0.14[Bc]	0.01 ± 0.00[A]
Succinate	0.6 ± 0.18[a]	0

[*] Different capital and small superscript letters indicate statistically significant differences ($p < 0.05$) among treatments within a line and a column, respectively

biodegradation in KT5. Generally, the numbers, positions and types of the substituted atoms of substituted aromatic compounds affect the microbial transformation. The biodegradation rates seemed to be controlled by the steric properties [39].

The previous report illustrated that glucose was essential for *Pseudomonas* sp. strain JS6 to degrade 4CT [27]. Succinate used at a common concentration (10 mM) led to repression of toluene catabolism transcription in *P. putida* mt-2 batch cultures, but the use of a low succinate concentration (0.25 mM) enhanced the pollutant removal efficiency [40]. In KT5 case, these phenomena were not apparent. The addition of succinate and yeast extract stimulated the utilization rates of KT5. Yeast extract serves as a carbon and nitrogen source for microorganisms. It contains growth factors, such as vitamins, which are probably essential for the activities of bacteria to enhance the degradation rates.

In comparison with chlorotoluenes, the utilization rates of chlorinated benzoates with the same chlorine numbers and positions were comparably faster. Obviously, benzoates were intermediates of biodegradation of corresponding toluenes by KT5, and their toxicities are lower than toluenes.

The abiotic and biotic stresses in a new environment may cause the fail in the introduction of new bacteria into soil due to their poor survival or low activity. In this study, the loss of the chemicals in the controls without bacteria inoculated was probably because of the degradation by indigenous microorganisms and adsorption by soil organic matter. Non-sterile soil inoculated with strain KT5 resulted in higher mineralization of toluenes compared with the sterile soil, which indicated that the bacterial strain could adapt to the complicated soil and cooperate with indigenous microorganisms well. The dissipation of toluene and chlorinated toluenes in non-sterilized soil was higher than in sterilized soil probably because of the degradation by indigenous microorganisms. The degradation rates of toluene in liquid media and in soil were higher than 3CT and 34DCT. The lower toxicity of toluene probably accounted for its higher degradation rate. However, the degradation rates in soil were comparably slower than that in the liquid media. The cell activities in soil can be influenced by the physicochemical properties of the soil, such as available nutrients, pH, oxygen concentration and other environmental factors. Although some reports on the biodegradation of toluene in soil are presented [38, 41–44], no study describing the biodegradation of chlorinated toluenes contaminated soil has been done so far.

There are some reports on the degradation pathway of toluene and substituted toluenes. The analysis of enzymes involving the degradation pathway not only reveals the degradation pathway, illuminates the inside degradation mechanisms of cells, but also determine the capacity of

enzyme activities, which may be a useful tool in the application of bacteria for production of enzymes. In a previous report, the TOL upper degradation pathway of toluene was described in detail in *P. putida* mt-2 [31, 34]. Catechols produced from the degradation of toluene and substituted toluenes by *P. putida* mt-2 were transformed via *meta* pathway [34]. The fungus *Cladosporium sphaerospermum* degraded toluene via the side chain pathway and then ring cleavage via *ortho* pathway [45]. The degradation several dichlorotoluenes by *Ralstonia* sp. PS12 took place via both upper and lower pathways [28]. Nevertheless, PS12 could not completely mineralize dichlorobenzoates, intermediates of chlorotoluenes degradation, in upper pathway. PS12 produced chlorocatechol 1,2-dioxygenase to mineralize dichlorotoluenes in lower pathway, not upper pathway. 2CT was known as a highly recalcitrant compound and was not completely degraded in previous reports [3, 46]. In this study, 2CT was completely degraded by KT5. It can be concluded that the bioaugmentation with *C. testosterone* KT5 could be an effective strategy to remediate the toluene, benzoate and their chlorine derivatives.

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