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ORIGINAL ARTICLE

Degradation of chlorotoluenes and chlorobenzenes by the dual-species biofilm of *Comamonas testosteroni* strain KT5 and *Bacillus subtilis* strain DKT

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Abstract

The cooperation of *Bacillus subtilis* strain DKT and *Comamonas testosteroni* KT5 was investigated for biofilm development and toluenes and chlorobenzenes degradation. *Bacillus subtilis* strain DKT and *C. testosteroni* KT5 were co-cultured in liquid media with toluenes and chlorobenzenes to determine the degradation of these substrates and formation of dual-species biofilm used for the degradation process. *Bacillus subtilis* strain DKT utilized benzene, mono- and dichlorinated benzenes as carbon and energy sources. The catabolism of chlorobenzenes was via hydroxylation, in which chlorine atoms were replaced by hydroxyl groups to form catechol, followed by ring fission via the *ortho*-cleavage pathway. The investigation of the dual-species biofilm composed of *B. subtilis* DKT and *C. testosteroni* KT5 (a toluene and chlorotoluene-degrading isolate with low biofilm formation) showed that *B. subtilis* DKT synergistically promoted *C. testosteroni* KT5 to develop biofilm. The bacterial growth in dual-species biofilm overcame the inhibitory effects caused by monochlorobenzene and 2-chlorotoluene. Moreover, the dual-species biofilm showed effective degradability toward the mixture of these substrates. This study provides knowledge about the commensal relationships in a dual-culture biofilm for designing multispecies biofilms applied for the biodegradation of toxic organic substrates that cannot be metabolized by single-organism biofilms.

Keywords *Bacillus subtilis* DKT · Chlorobenzenes · *Ortho*-cleavage pathway · *Comamonas testosteroni* KT5 · Dual-species biofilm · Biodegradation

Introduction

Chlorobenzenes are components widely used to produce a number of products such as solvent, pesticides, dyes, pharmaceuticals, rubbers, plastics, and disinfectants (Yadav et al. 1995; Zhang et al. 2005). Because of the widespread and extensive uses, they have been commonly detected in environments, including in air (Popp et al. 2000), soil (Wang et al. 1995; Zolezzi et al. 2005), water (Nikolaou et al. 2002; Monferrán et al. 2005), and sediments (Lee et al. 2005). Moreover, chlorobenzenes are absorbed into vegetables (Wang and Jones 1994), which may harmfully affect people and other species in food chains. These compounds are toxic and persistent, so they are identified as priority pollutants by the US Environmental Protection Agency (Zhang et al. 2011).

In spite of easy evaporation and adsorption, biodegradation is a main mechanism for the removal of chlorobenzenes from wastewater (Namkung and Rittmann 1997). A number of bacterial pure cultures utilizing these substrates as carbon and energy sources have been reported. They are *Alcaligenes* sp. (Schraa et al. 1986), *Pseudomonas* sp. (van der Meer et al. 1987; Spain and Nishino 1987; Sander et al. 1991; Brunsbach and Reineke 1994; Potrawfke et al. 1998), *Xanthobacter flavus* 14p1 (Spiess et al. 1995) *Burkholderia* PS14 (Rapp

Highlights
Bacillus subtilis strain DKT degraded a wide range of chlorobenzenes.
The degradation pathway for chlorobenzenes in *B. subtilis* strain DKT was via hydroxylation, in which chlorine atoms were replaced by hydroxyl groups to catechol.
Bacillus subtilis DKT synergistically promoted *Comamonas testosteroni* KT5 in biofilm development.
The dual-species biofilm formed by *B. subtilis* DKT and *C. testosteroni* KT5 showed effective degradation of chlorotoluenes and chlorobenzenes.

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and Timmis 1999; Rapp 2001), *Rhodococcus* sp. and *Rhodococcus phenolicus* (Rapp and Gabriel-Jürgens 2003; Rehfuss and Urban 2005) and *Acidovorax avenae* (Monferrán et al. 2005). However, the chlorobenzenes disintegration is limited (Zhang et al. 2011).

In natural environments, chlorobenzenes normally do not exist alone but with other chemical compounds. Nikolaou et al. (2002) showed that river water, lake water, seawater, and treated wastewater in Greece were contaminated with a number of organic compounds, and most of them were chlorobenzenes and chlorotoluenes. The presence of xenobiotics may affect the remediation of target compounds. For examples, the toluene and benzene biodegradation inhibited each other resulting in reducing the remediation rates (Chang et al. 1993; Oh et al. 1994; Bielefeldt and Stensel 1999; Reardon et al. 2000; Lin et al. 2007). Thus, the use of microbial consortia should be investigated to overcome the inhibition. However, microorganisms in a binary mixture may compete and inhibit each other, which reduces the degradation efficiency.

Biofilm is a natural immobilization method preferred for eliminating toxic chemicals in water and in gas stream because microorganisms can tolerate the unfavorable conditions such as exposure to toxic chemicals and environmental stresses. In the natural ecosystems, microbes with strong biofilm may function as a bridging organism which co-aggregates with other microorganisms to develop biofilms (Rickard et al. 2002, 2004). However, the biodegradability toward chlorobenzenes and chlorotoluenes by biofilm has not been extensively investigated. Moreover, the microbial cooperation in biofilm illustrating the process in natural conditions has not been illuminated.

In a previous report, *Comamonas testosteroni* KT5 effectively utilized a wide range of chlorinated toluenes as carbon and energy sources (Duc 2017). However, the isolate showed low biofilm formation. On the other hand, *Bacillus subtilis* DKT, a new chlorobenzene-degrading bacterium isolated from a contaminated site, was a high biofilm formation strain. In this paper, the cooperation of both isolates was investigated for biofilm development and the degradation of toluenes and chlorobenzenes.

Methods

Culture media

The mineral medium (MM medium) components were described by Duc (2017). Solid medium was obtained by adding 2% agar (*w*/*v*). The MM medium was supplemented with 0.1% yeast extract (*w*/*v*, MMY medium) used in biofilm experiments. The media were autoclaved at 121 °C for 15 min. Benzene, monochlorobenzene, dichlorobenzenes, chlorotoluenes, and other organic compounds (purity >9 9.5%) were used. All chemicals were purchased from Sigma-Aldrich (Singapore) or Merck (Germany).

Enrichment, isolation, and identification of the bacterial strain

Soil, mud, river sediment, and sewage sludge samples were collected at the same sites as described previously (Duc 2017). Samples (5 g) were dispensed in a 500-mL flask containing 200 ml of MM medium. The enrichment culture was incubated at room temperature (approximately 30 °C) and 150 rpm for 1 month. Then, 0.5 mM monochlorobenzene was supplemented every week. Sample solution was diluted and spread on solid MM medium supplemented with 0.5 mM monochlorobenzene as a sole carbon and energy source to obtain single colonies. Single strains were transferred to liquid media supplemented with benzene and chloro-substituted benzenes to examine their growth and biodegradability. The obtained isolate was identified genetically as described in the earlier report (Duc 2017). The 16S rRNA gene sequence was compared with other sequences available in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and identified using Ribosomal Database Project (RDP; http://rdp.cme.msu.edu).

Chemical degradation by freely suspended cells

The degradation experiments by suspended bacteria were conducted in the MM medium. Cosubstrates (glucose, succinate, citrate, and yeast extract) were added at 0.1% (*w*/*v*). Bacteria grew in liquid LB medium for 18 h with approximately 2×10^8 CFU/ml used for inoculation, and 0.5 ml of which was added to 100 ml of respective media. For chemical degradation by the mixture, each strain was cultivated with the same cell numbers at the beginning (10^6 CFU/ml in total). Benzenes were added at various concentrations, and 2-chlorotoluene was used at 1.0 mM. The biodegradation by suspended cells was determined at room temperature with a shaking speed of 150 rpm. Liquid samples (2.0 ml) were collected at specific times to determine the cell growth and remaining chemicals.

Biofilm formation assay

The biofilm formation was conducted in the culture system comprising new 150-ml cylinder bottles containing 50 ml of MMY medium and seven pieces of microscope glass slides (24.5 × 76.2 × 0.8 mm). The glass slides were separately embedded into plastic devices stuck to the cover which kept the pieces not to clash during shaking. Then, 3/4 of the glass slides were submerged in liquid media when the bottles were in the static condition. Bacteria were suspended in media with the same cell number as described above. The incubation process was carried out at room temperature and 60-rpm shaking speed.

The biofilm was quantitated based on the viable counts of cells attached to the glass slides. Every 6-h period, a number

of bottles with glass slides were used to determine biofilm formation, while others were continued to do the experiment. The slides were gently washed three times with sterilized saline solution (0.85% NaCl), placed in a sterile glass tube containing 10 ml of sterile saline solution, and then vortexed for 10 min to release cells from biofilm. A wooden device was also used to scrape the remaining attached cells. The solution was serially diluted and spread onto LB medium agar. Cell numbers of each strain were counted based on the morphological features of emerging colonies. *C. testosteroni* KT5 formed round and convex colonies, while colonies of *B. subtilis* DKT were irregular, large size, and undulate margin.

Biofilm biomass and chemical degradation in the biofilm-batch reactors

The medium in the bottles was removed after 24 h. The bottles and slides were rinsed in triplicate with sterilized saline solution. The bottles were then filled with a new MMY medium and incubated. The same operation was repeated in the following intervals (24 h each). Monochlorobenzene and 2-chlorotoluene in media were 0.5 and 1.0 mM, respectively. The controls without bacteria and with dead cell biofilm were run in parallel. The dead cell biofilm was obtained by sterilizing the biofilm slides with ethanol (70%, *v*/*v*).

Analytical methods

Chemicals were extracted from liquid media with equal volume of dichloromethane three times. The solutions were evaporated to dryness under a nitrogen stream and then dissolved in absolute methanol. The chemical concentrations remaining during degradation process were determined using HPLC Model 600E with spherisorb C18 5 UV, 4.5 × 250 mm column, which methanol was the mobile phase at the flow rate of 0.5 ml/min. The Waters UV detector model 2487 was used at 254 nm. Moreover, gas samples (1.0 ml) in headspace were periodically taken for GC (Agilent 6850) to determine the remaining chemicals.

Degradation pathway of chlorobenzenes in *B. subtilis* DKT

The analysis of derivative metabolites was carried out using gas chromatography–mass spectrometry (GC-MS) (Agilent 7890N/MS 5975), which was equipped with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The oven temperature was 100 °C for 1 min, ramped to 280 °C at 5 °C/min, and held for 10 min. The temperatures of injector, interface, and the ionization source were 180, 280, and 250 °C, respectively. The mass spectra were obtained at 70 eV. The results of HPLC and GC-MS were compared with retention times and ion spectra of authentic standards.

For determination of enzymes involved in degradation pathways, cells grew in MMY medium supplemented with 0.1 mM benzene and chlorinated benzenes for 24 h. Bacteria were harvested by centrifugation for 5 min at 12,000 rpm, then washed twice and resuspended in a sterilized saline solution (0.85% NaCl). The cells were disrupted by sonication for 5 min, and then centrifuged at 12,000 rpm for 10 min. The supernatant was collected to measure enzyme activities. The activities of cis-1,2-dihydroxycyclohexa-3,5-diene dehydrogenase (dihydrodiol dehydrogenase); catechol 1,2-dioxygenase; and catechol 2,3-dioxygenase were performed based on a previous report (Spain and Nishino 1987).

Statistical analysis

All experiments were conducted at least in triplicate. Data were shown as the means ± standard deviation. The SPSS software program version 22.0 was used to perform analysis of variance, and significant differences ($p < 0.05$) were calculated using Duncan's test.

Results

Isolation and identification of the benzene and chlorobenzene-degrading strain

DKT was isolated from soil utilizing benzene and chlorinated benzene as sources of carbon and energy. The 16S rRNA sequence showed the highest degree of nucleotide identity with *B. subtilis* isolates (99% identity) of the sequences available in the NCBI GenBank database and Ez-Biocloud. The RDP analysis also indicated that the strain belongs to genus *Bacillus*. The obtained 16S rDNA has been deposited in the GenBank under accession number MH109504.1. The strain DKT has been also deposited in the Culture Collection at the Center for Biochemical Analysis (Vietnam) under the deposition number DUCOANH2015-6A.

Biodegradation of benzene and chlorinated benzenes in liquid culture by freely suspended *B. subtilis* DKT

Bacillus subtilis DKT utilized benzene, monochlorobenzene, and dichlorobenzenes with different rates (Fig. 1). Benzene was degraded with the highest rate, followed by 1,2-dichlorobenzene, while the degradation rate of 1,3-dichlorobenzene was lowest. Bacteria grew with higher rates in the medium containing substrates with higher degradation rates. *Bacillus subtilis* DKT degraded 1,2-dichlorobenzene quite effectively compared to other compounds. In the MM medium with 1,2-dichlorobenzene, the isolate could grow up to 1.0 mM, and completely inhibited to grow at 1.2 mM (Fig. 2).

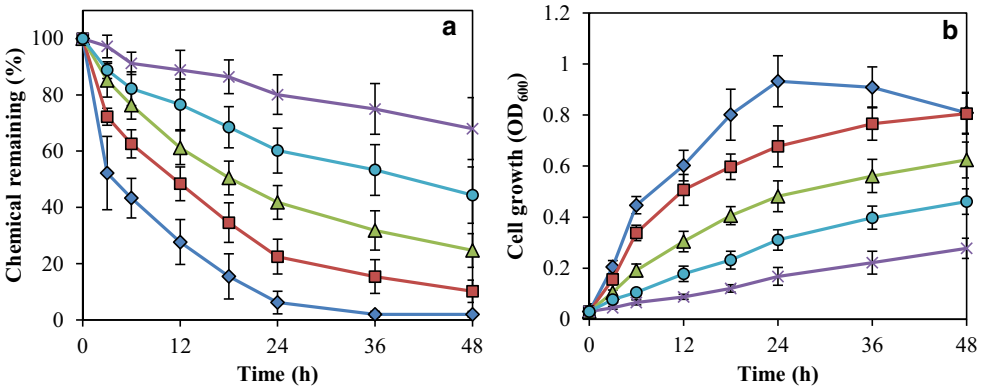

Fig. 1 Degradation of individual benzene and chlorinated benzenes (**a**) as sole carbon sources by *B. subtilis* DKT and bacterial growth in the medium (**b**). Benzene (filled diamonds), 1,2-dichlorobenzene (filled squares), monochlorobenzene (filled triangles), 1,3-dichlorobenzene (crosses), and 1,4-dichlorobenzene (filled circles) were added at 0.3 mM

Effects of monochlorobenzene and cosubstrates on degradation rates and biofilm formation of *B. subtilis* DKT

Table 1 presents that the degradation rates were highest at 0.1 mM monochlorobenzene and the increment of initial concentrations resulted in the decrease of degradation rates. The supplementation of any cosubstrates stimulated degradation rates, cell numbers in both biofilm and the suspended counterpart. The monochlorobenzene degradation rates were highest with the presence of glucose. However, the biofilm levels and a number of planktonic bacteria were not statistically different among treatments with the same chemical concentrations and various exogenous carbon sources (Table 1).

Formation of individual and dual-species biofilms of *B. subtilis* DKT and *C. testosteroni* KT5

The degradation rates of 2-chlorotoluene by *C. testosteroni* KT5 and monochlorobenzene by *B. subtilis* DKT were moderate compared to other isomers, so they were selected as additional substrates in these experiments. The effects of monochlorobenzene on biofilm formation were determined at 0.5 mM. Because *C. testosteroni* KT5 degraded chlorotoluenes so quickly at 0.5 mM, the biofilm experiments of this strain were conducted at 1.0 mM 2-chlorotoluene and different monochlorobenzene concentrations. The determination of individual and dual-species biofilms was carried out during the cell growth with the presence of chemical compounds. The yeast extract addition in liquid media stimulated the degradation of toluene and chlorinated toluenes by *C. testosteroni* KT5 (Duc 2017). Yeast extract also stimulated biofilm development and monochlorobenzene degradation by *B. subtilis* DKT in liquid media. The presence of yeast extract in media resulted in higher degradation rates of toluenes by *C. testosteroni* KT5 compared to the presence of glucose. Therefore, yeast extract was added as a cosubstrate in these experiments. The supplementation of monochlorobenzene and 2-chlorotoluene from 0.1 to 1.0 mM inhibited the growth of both freely suspended and biofilm cells of individual cultures (Fig. 3).

The biofilm formation of each isolate in MMY medium without both toluenes and benzenes was also carried out. In this medium, the attached cells of *B. subtilis* DKT on a glass slide was $12.1 \times 10^7 \pm 1.3 \times 10^7$ CFU, while the figures for *C. testosteroni* KT5 were $3.3 \times 10^4 \pm 0.4 \times 10^4$ CFU per slide. These results indicated that biofilm-forming capacity of *B. subtilis* DKT significantly higher than that of *C. testosteroni* KT5. Figure 3 also illustrates the differences of biofilm levels between two strains.

The biofilm formation of the mixed-species culture growing on both monochlorobenzene and 2-chlorotoluene was

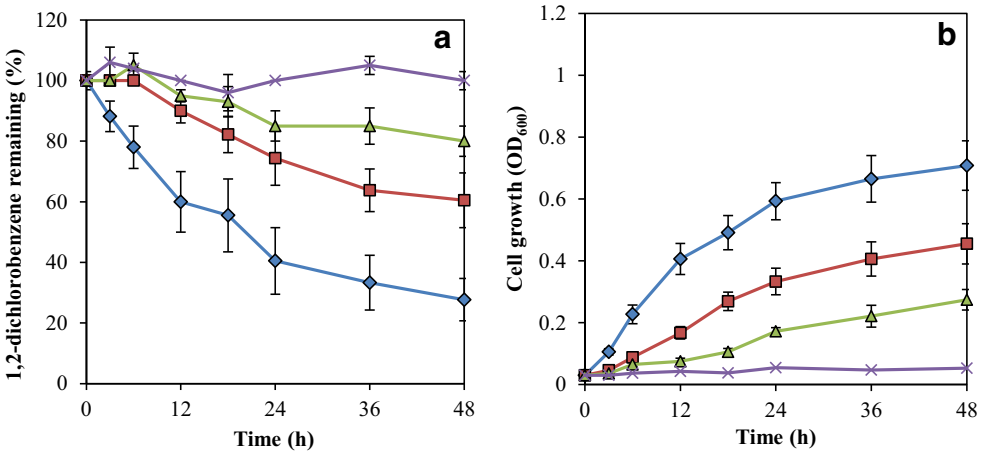

Fig. 2 Degradation of 1,2-dichlorobenzene (**a**) as a carbon source by *B. subtilis* DKT and cell growth in the medium (**b**) at 0.5 mM (filled diamonds), 0.8 mM (filled squares), 1.0 mM (filled triangles), and 1,2 mM (crosses)

Table 1 Effects of monochlorobenzene concentrations and cosubstrates on degradation rates and biofilm formation of *B. subtilis* DKT cultured in media for 24 h

Monochlorobenzene (mM)	Cosubstrates*	Degradation (%)**	Freely suspended cells (× 107 CFU/ml)**	Biofilm formation (× 107 CFU/slide)**
0.1	None	77.2 ± 7.0d	5.7 ± 0.5a	10.4 ± 0.9a
	Glucose	97.4 ± 1.7f	10.1 ± 0.6c	21.7 ± 2.0bc
	Succinate	88.7 ± 5.8ef	10.5 ± 0.7c	23.9 ± 2.3c
	Citrate	90.5 ± 5.4ef	10.7 ± 1.1c	26.1 ± 1.9c
	Yeast extract	94.4 ± 3.8ef	9.8 ± 0.9c	24.2 ± 2.1c
0.3	None	45.1 ± 14.3b	6.1 ± 0.4ab	14.3 ± 1.0ab
	Glucose	86.4 ± 8.8e	27.7 ± 2.4d	60.0 ± 6.1d
	Succinate	76.9 ± 9.3d	30.1 ± 4.0d	64.2 ± 10.7de
	Citrate	71.0 ± 9.0d	29.4 ± 2.5d	68.7 ± 6.3e
	Yeast extract	74.8 ± 9.3d	27.8 ± 2.6d	63.0 ± 7.5de
0.5	None	12.9 ± 3.2a	5.2 ± 0.6a	9.2 ± 1.1a
	Glucose	50.4 ± 12.6c	8.8 ± 0.9bc	20.1 ± 1.1bc
	Succinate	41.5 ± 12.6b	9.4 ± 0.5c	22.9 ± 2.0c
	Citrate	38.9 ± 11.7b	9.1 ± 0.6c	22.4 ± 1.2c
	Yeast extract	40.3 ± 6.3b	8.8 ± 0.6bc	21.7 ± 2.3bc

*Cosubstrates were added at 0.1%

**Different letters (a–f) indicate statistically significant differences among treatments within a column ($p < 0.05$)

shown in Fig. 4. At this condition, a cell number of both strain in the mixed-species biofilm were significantly higher than those in the single-culture biofilms (shown in Fig. 3).

Formation of single and dual-species biofilms of *B. subtilis* DKT and *C. testosteroni* KT5 and application for 2-chlorotoluene and monochlorobenzene degradation

The biofilm formation and chemical degradation by individual and dual-species biofilms were determined with the addition of 2-chlorotoluene or/and monochlorobenzene. Biofilm levels increased in the following cycles in general. In the media, with the presence of 2-chlorotoluene (1.0 mM), a cell number in biofilm of *B. subtilis* DKT were significantly lower compared to those in biofilm developing in the media without 2-chlorotoluene in all circles ($p < 0.05$) (Fig. 5a, b). Similarly, monochlorobenzene (0.5 mM) inhibited the biofilm formation as well as the degradation rates of *C. testosteroni* KT5 (Fig. 6a). The low biofilm formation due to the addition of a xenobiotic chemical resulted in the low degradation rates, which showed the evidence that single-species biofilms could not effectively remediate any substrate in the mixture of the contaminated compounds.

The settled bacteria in the dual-species biofilm were shown in Fig. 7. In the media with the presence of both exogenous toxic compounds, the attached cells of any strain in the dual-species biofilm were significantly higher compared to single-

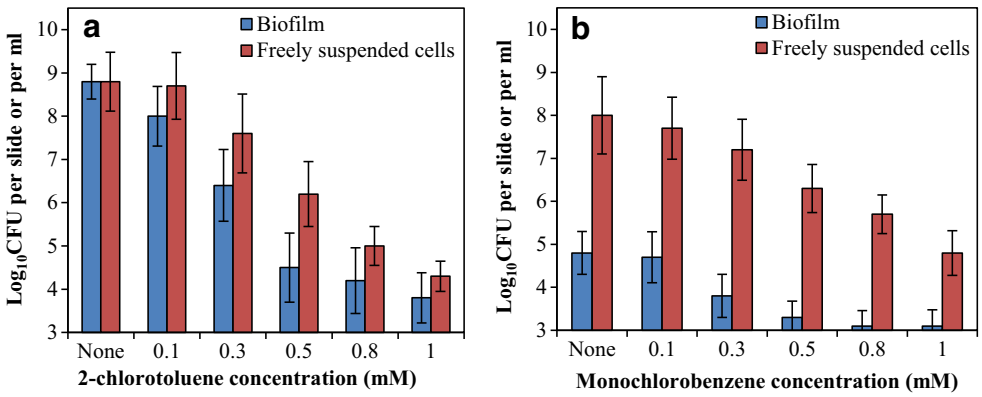

Fig. 3 Cell numbers of *B. subtilis* DKT (**a**) and *C. testosteroni* KT5 (**b**) growing in the single-species cultures after 24 h of incubation. The biofilm formation of DKT was carried out in MMY medium supplemented with 0.5 mM monochlorobenzene and various 2-chlorotoluene concentrations, while the biofilm formation of KT5 was conducted at 1.0 mM 2-chlorotoluene and various monochlorobenzene concentrations

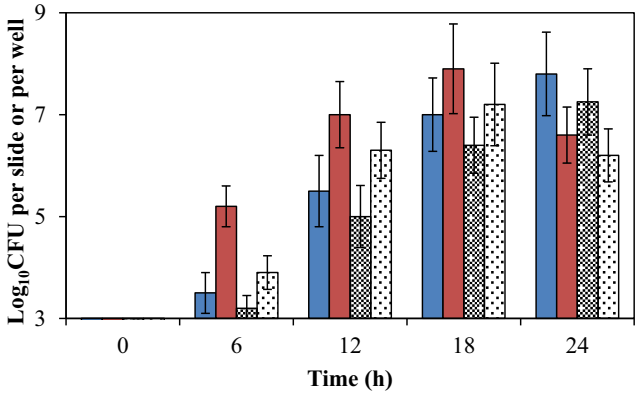

Fig. 4 Freely suspended cell numbers of *B. subtilis* DKT (▭) and *C. testosterone* KT5 (▭) and attached cells numbers of *B. subtilis* DKT (▭) and *C. testosterone* KT5 (▭) in the mixed-species culture culturing in MMY medium supplemented with both 2-chlorotoluene (1.0 mM) and monochlorobenzene (0.5 mM)

species ones presented in Fig. 5a and 6a ($p < 0.05$). The increase of cell numbers in mixed-culture biofilms resulted in

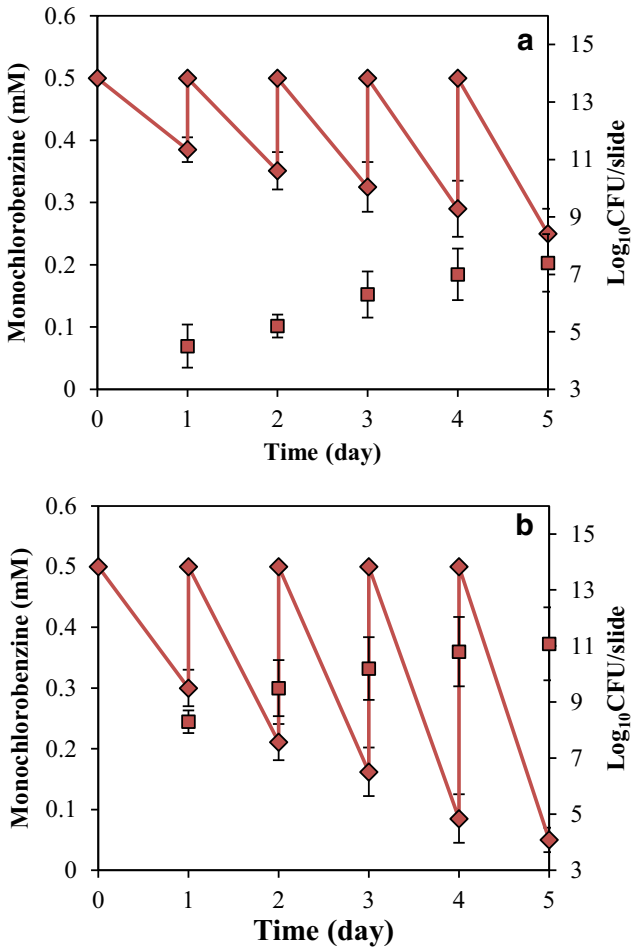

Fig. 5 Cell numbers in single-species biofilms (filled squares) of *B. subtilis* DKT and monochlorobenzene degradation (filled diamonds) by the bacterial strain. The experiments were carried out using MMY medium supplemented with 1.0 mM 2-chlorotoluene (**a**) and without 2-chlorotoluene (**b**)

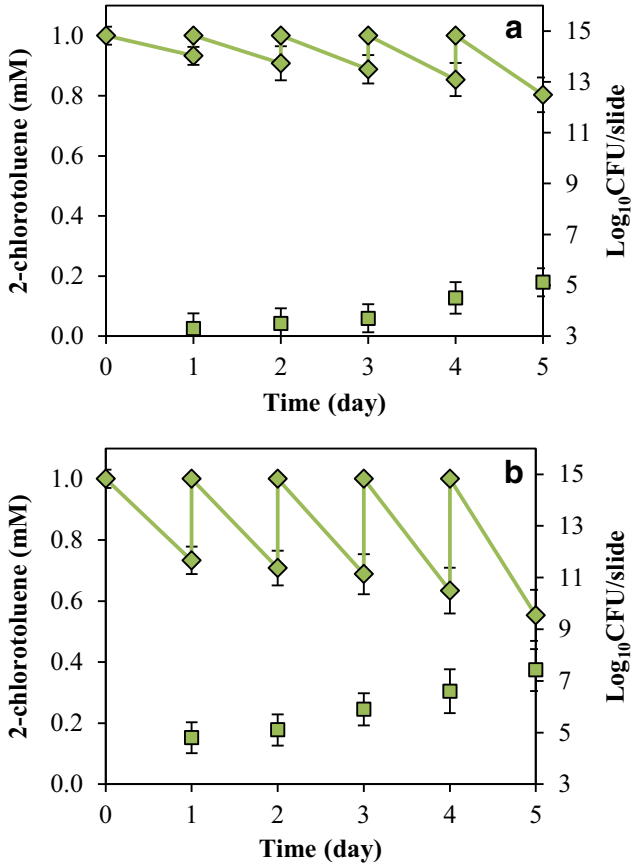

Fig. 6 Cell numbers in single-species biofilms (filled squares) of *C. testosteroni* KT5 and 2-chlorotoluene degradation (filled diamonds) by the bacterial strain. The experiments were carried out using MMY medium supplemented with 0.5 mM monochlorobenzene (**a**) and without monochlorobenzene (**b**)

the enhancement of degradation rates (Fig. 7). *Comamonas testosteroni* KT5 and *B. subtilis* DKT fixed in the dual-species biofilm in the fifth circle were around 1.6×10^9 CFU and 2.5×10^{10} CFU on a slide, which were almost 1.2×10^4 and 0.1×10^4 times as many as those in single-species biofilms in the same media, respectively. The biofilm levels of *B. subtilis* DKT in the mixed-culture biofilm (Fig. 7b) were not statistically different from the single-culture biofilm forming in media without 2-chlorotoluene shown in Fig. 5b. By contrast, cell numbers of *C. testosteroni* KT5 settled in the mixed-species biofilm (Fig. 7a) were almost 60 times as many as cells in the single-species biofilm in the fifth circle shown in Fig. 6b. Meanwhile, the chemicals lost in all controls were negligible (data were not shown).

Degradation pathway of chlorobenzenes in *B. subtilis* DKT

During the monochlorobenzene degradation, transient 2-chlorophenol (*m/z* 128, 130, 64) was detected. In another experiment, 1,2-dichlorobenzene was transformed to 2-

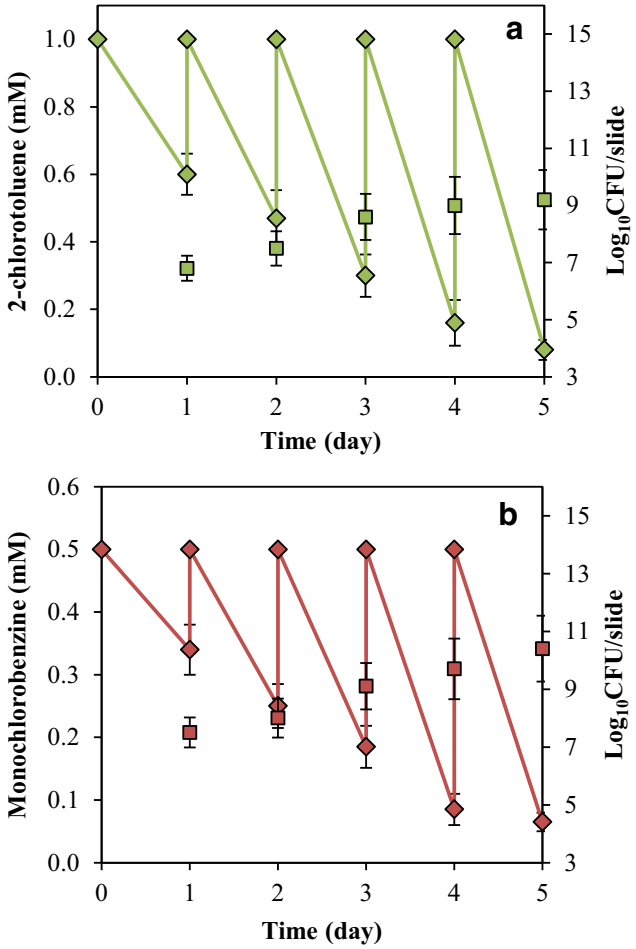

Fig. 7 Cell numbers in mixed-species biofilm (filled squares) and chemical degradation (filled diamonds). 2-chlorotoluene degradation and the number of *C. testosteroni* KT5 (**a**, green color), and monochlorobenzene degradation and the number of *B. subtilis* DKT (**b**, red color) in mixed-species biofilm were shown

chlorophenol and catechol. These results suggested that the hydroxylation processes occurred during the degradation.

To determine further degradation pathways, enzymes involved in the degradation were determined (Table 2). Enzymes were extracted from bacteria growing on the medium containing benzene and chloro-substituted benzenes for activity measurement in comparison with the cells cultured on only yeast extract. The activities of enzymes extracted from cells growing on yeast extract were significantly lower than those from bacteria growing on benzenes. The activities of dihydrodiol dehydrogenase extracted from cells cultured on benzene, monochlorobenzene, and 1,2-dichlorobenzene were similar. The activities of catechol 1,2-dioxygenase induced in benzene and chlorobenzene-grown cells were also confirmed in bacteria toward catechols. The catechol 1,2-dioxygenase produced from mixed-species cells in the batch culture of biofilm was active, while the activities of catechol-2,3-dioxygenase were negligible in both single and dual species, suspended cells and biofilms (data were not shown). This result proved that the substrates degradation via the *ortho*-cleavage pathway. Accordingly, the degradation pathways of monochlorobenzene and 1,2-dichlorobenzene are proposed in Fig. 8.

Discussion

Bacillus subtilis DKT utilized benzene, mono-, and all dichlorobenzene as sole carbon and energy sources. However, the isolate could not degrade any chlorinated toluenes. The increase of benzene concentrations resulted in lower degradation rates due to the increase of toxicity, but cell growth and biofilm levels were highest at 0.3 mM because bacteria had a suitable carbon source. In previous reports, some strains of genera *Bacillus* could degrade benzene and some chlorinated benzenes (Wang et al. 2003; Liu et al. 2010; Mukherjee and Bordoloi 2012; Shadi et al. 2015; Vyas and Murthy 2015). Some other microorganisms were also reported not to degrade all isomers of dichlorobenzenes (Schraa et al. 1986; Haigler et al. 1988; van der Meer et al. 1991; Spiess et al. 1995; Zhang et al. 2011). For example, *Ralstonia pickettii* L2 (Zhang et al. 2011) and *Alcaligenes* sp. strain A175 (Schraa et al. 1986) could degrade benzene, monochlorobenzene, and 1,3- and 1,4-dichlorobenzene but could not degrade 1,2-dichlorobenzene. The degradation rates of each isomer among

Table 2 Activities of enzymes extracted from *B. subtilis* DKT growing on benzene, chlorinated benzenes and yeast extract

Enzyme	Assay substrates	Specific enzyme activities (μmol/min/mg of protein) extracted from bacteria grew on substrates and yeast extract			
		Benzene	Chlorobenzene	1,2-dichlorobenzene	Yeast extract
Diol dehydrogenase	Benzene dihydrodiol	0.307 ± 0.045	0.261 ± 0.034	0.275 ± 0.032	0.026 ± 0.015
	1,2-dichlorobenzene dihydrodiol	0.205 ± 0.026	0.178 ± 0.017	0.187 ± 0.015	0.017 ± 0.008
Catechol 1,2-dioxygenase	Catechol	0.242 ± 0.027	0.186 ± 0.022	0.207 ± 0.031	0.035 ± 0.012
	3-Chlorocatechol	0.106 ± 0.011	0.184 ± 0.031	0.177 ± 0.027	0.026 ± 0.011
	4-Chlorocatechol	0.082 ± 0.012	0.165 ± 0.020	0.148 ± 0.011	0.011 ± 0.005
Catechol 2,3-dioxygenase	Catechol	< 0.001	< 0.001	< 0.001	0

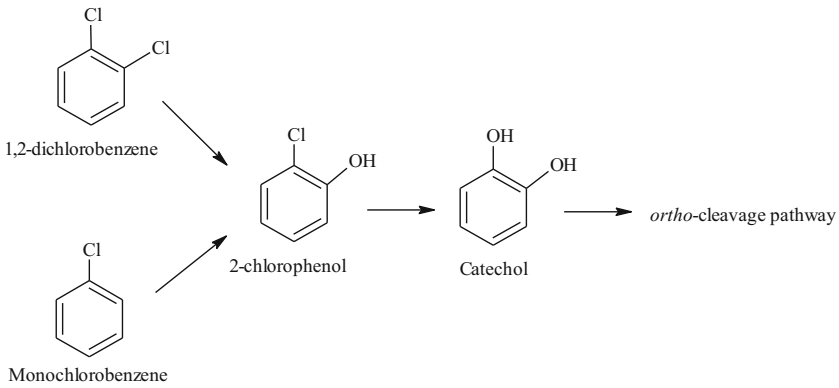

Fig. 8 Proposed biodegradation pathways of chlorobenzenes in *B. subtilis* DKT

chlorobenzenes depended on different strains. For example, *Pseudomonas* sp. JS100 seemed to degrade 1,2-dichlorobenzene with a higher rate than monochlorobenzene and other dichlorobenzenes (Haigler et al. 1988). However, *A. avenae* degraded monochlorobenzene and all dichlorobenzenes with similar rates (Monferrán et al. 2005). The rates of degradation of these compounds by *B. subtilis* DKT were benzene > 1,2-dichlorobenzene > monochlorobenzene > 1,4-dichlorobenzene > 1,3-dichlorobenzene. The utilization of 1,2-dichlorobenzene as a sole carbon source has been presented in some reports (van der Meer et al. 1987; Haigler et al. 1988; Ziagova and Liakopoulou-Kyriakides 2007). *Pseudomonas* sp. could grow on and degrade 1,2-dichlorobenzene up to 1.0 mM, but it required glucose in media (Ziagova and Liakopoulou-Kyriakides 2007).

During 1,2-dichlorobenzene degradation by *B. subtilis* DKT, chlorine atoms were first replaced by hydroxyl groups to 2-chlorophenol and then catechol. Dichlorocatechols were common metabolites produced during biodegradation of dichlorobenzenes (Schraa et al. 1986; Spain and Nishino 1987; Haigler et al. 1988; Sander et al. 1991; Monferrán et al. 2005; Ziagova and Liakopoulou-Kyriakides 2007; Zhang et al. 2011). In other studies, 1,2-dichlorobenzene was transformed by *Pseudomonas* sp. and *Staphylococcus xylosus* to 3,4-dichlorocatechol and then ring fission via *ortho*-cleavage pathway (Haigler et al. 1988; Ziagova and Liakopoulou-Kyriakides 2007). The replacement of a chlorine atom by a hydroxyl group was reported in monochlorobenzene degradation (Zhang et al. 2011). However, no previous study showed the replacement of both chlorine atoms in dichlorobenzenes to catechol similar to *B. subtilis* DKT. The investigation of enzymes involving in the degradation pathway indicated that all tested compounds were degraded via the *ortho*-cleavage pathway. The *ortho*-cleavage pathway for chlorobenzene degradation was also described in previous studies (Schraa et al. 1986; Nishino et al. 1992; Spiess et al. 1995; Zhang et al. 2011).

The use of biofilm for biodegradation is efficient, environmentally friendly, safe, and cheap (Pandey and Jain 2002; Paul et al. 2005). The settlement of microorganisms on specific surfaces depends on microorganisms, nutrients, and other environmental conditions. In natural environments, microbes usually exist in communities and most biofilms are composed of a number of microbial species (Perumbakkam et al. 2006; Singh et al. 2006; Giaouris et al. 2013; Makovcova et al. 2017), and they may cooperate, compete, or have neutral interactions (Giaouris et al. 2013; Makovcova et al. 2017). However, the commensal relationship in multi-species biofilms has not been intensively investigated.

The biofilm development of *B. subtilis* DKT and *C. testosteroni* KT5 was affected by exogenous toxic chemicals and cosubstrates. The addition of cosubstrates enhanced the degradation rates of monochlorobenzene and biofilm formation of *B. subtilis* DKT due to the increase of suspended cells in liquid media. However, the toxic substrates inhibited the biofilm development in this study. Both *B. subtilis* DKT and *C. testosteroni* cultured separately were inhibited to grow in liquid and inhibited to develop biofilms when both 2-chlorobenzene and monochlorobenzene presented in the media. On the other hand, no obvious inhibitory effects on the growth of both strains occurred in the mixed culture. Each isolate in the mixed-species biofilm continuously developed from the first to the fifth circles, and the biofilm levels were significantly higher than the single-species forms even though both monochlorobenzene and 2-chlorotoluene were added into media. These results might be because the chemicals were degraded by the binary mixture to reduce concentrations, and *C. testosteroni* KT5 was synergistically promoted by *B. subtilis* DKT. In a previous study, some chemical-degrading bacteria with weak biofilm-forming capacity were cultured with other isolates with high biofilm formation in synergistic promotion to enhance the settlement and degradation (Li et al. 2008). Acetonitrile-degrading bacterium *Rhodococcus rhodochrous* BX2 was co-cultured with several non-degrading bacteria with higher biofilm to stimulate BX2 attachment (Li et al. 2013). Yoshida et al. (2009) presented that chlorobenzoate-degrading *Burkholderia* sp. NK8 (low biofilm formation) was synergistically adhered on a surface with *Pseudomonas aeruginosa* PAO1 to stimulate the settlement of NK8.

In our study, the activities of the dual-species culture were significantly higher than those carried out solely by individual

isolates. The degradation of a target substrate and the biofilm formation of individual isolates were inhibited by the presence of an exotic compound. *C. testosteroni* KT5 co-cultured with *B. subtilis* DKT increased the settlement of both strains on the surface and enhanced the degradation rates. The biofilm formation relating to interspecies cell signaling has been demonstrated for a variety of microorganisms (Bassler et al. 1997). In a previous study, *Pseudomonas* sp. GJ1 (a 2-chloroethanol-degrading bacterium) and *Pseudomonas putida* DMP1 (a *p*-cresol degrading organism) established a commensal relationship in biofilm development, and DMP1 mitigated the inhibitory effects of *p*-cresol on *Pseudomonas* sp. GJ1 (Cowan et al. 2000). In the mixed-species biofilms, bacteria were protected against a toxic chemical better than a single-species one (Leriche et al. 2003). These results demonstrated that degrader bacteria can be co-cultured, which enables them to develop a mixed microbial flora and enhance their degradation capacities. The incorporation of *C. testosteroni* KT5 and *B. subtilis* DKT should be applied to biodegrade benzene, toluene, and these chlorinated compounds which co-contaminate in water (Slaine and Barker 1990; Nikolaou et al. 2002; Groschen et al. 2004; Mottaleb et al. 2003; Robinson et al. 2004; Waddell et al. 2004).

This study investigated the biodegradability of *C. testosteroni* KT5 (a chlorinated toluene-degrading strain) and *B. subtilis* DKT (a new chloro-substituted benzene-degrading strain) as well as the adaptability of the dual-species biofilm in the biodegradation of a mixture of organic xenobiotics. Two isolates commensally formed dual-species biofilm rather than competition, which is essential for reducing xenobiotics toxicity and stimulates multispecies microbial consortia for biological treatment. The cooperation of two isolates enhanced the biofilm formation and chemical degradation. Even though it was impossible to determine the ratios of substrates degraded by biofilm and by freely suspended cells in media, the mutual or commensal relationships among species in the mixed-culture biofilm should be investigated more for the remediation of mixed organic compounds.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Research involving human participants and/or animals This article does not involve human participants or animals performed by any of the authors.

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