Degradation of *p*-chlorocresol by facultative *Thauera* sp. strain DO

Duc Danh Ha¹ · Oanh Thi Nguyen¹

Received: 29 January 2019 / Accepted: 20 December 2019 / Published online: 11 January 2020
© King Abdulaziz City for Science and Technology 2020

Abstract

In this work, *Thauera* sp. DO isolated from sludge and sediment utilized *p*-chlorocresol and some related compounds as the sole carbon and energy sources under both aerobic and anaerobic conditions. The pathways for *p*-chlorocresol in the isolate under each condition were different. Under the aerobic condition, *p*-chlorocresol was degraded via two separate pathways. The first was the reductive dehalogenation reaction, in which the substrate was transformed to *m*-cresol followed by the catechol degradation pathway, and the second aerobic pathway for *p*-chlorocresol was the methyl oxidation to 4-chlorobenzoate. Under the anaerobic conditions, *p*-chlorocresol was rapidly dechlorinated in the first step to *m*-cresol, followed by several steps prior to the complete degradation. The determination of *p*-chlorocresol degradation in liquid media by whole cells showed that 100% and 85% of the substrate (0.3 mM) were transformed within 12 h under aerobic and anaerobic conditions, respectively, while nearly 100% of this compound was degraded within 6 h using the two-stage anaerobic–aerobic degradation process. These results show a novel method to increase the degradation rates of *p*-chlorocresol using the anaerobic process followed by the aerobic process.

Keywords *Thauera* sp. DO · *p*-Chlorocresol · Dehalogenation · Pathways · Anaerobic–aerobic degradation

Introduction

p-Chlorocresol (or 4-Cl-*m*-cresol, 4-chloro-3-methylphenol) is widely used as an antiseptic and preservative in pharmaceuticals. For example, it is used for prion decontamination of medical devices (Riemer et al. 2006), a fungicide, and microbiocide/microbiostat (USEPA 1997). The chemical is also used in the production of packaging materials, adhesives, glues, inks, cosmetics, paints and varnishes, textile finishes, leather and tanning agents, and industrial oils and emulsions (Dooms-Goossens et al. 1981). *p*-Chlorocresol has been widely detected in environments such as drinking water (Kool et al. 1982; Huang et al. 2003), surface water (Great Lakes Water Quality Board 1983), seawater (Kot-Wasik et al. 2004), effluents from a municipal wastewater treatment plant (Verschueren 2001), effluents from treatment

works, soil leachate, wastewater sludge (Buisson et al. 1984), sewage treatment (Stangroom et al. 1998), air effluent from a municipal waste incinerator (James et al. 1985), wastewater treatment plants (Schnaak et al. 1997; Verschueren, 2001), and sediments (Acharya and Ives 1994; Lopes and Furlong 2001).

p-Chlorocresol is a moderate allergen, an irritant to sensitive skin (Maibach 2001; Lewis 2007), and causes other human health problems (USEPA 1997; Andersen 2006). Moreover, the compound is corrosive to metals and forms complex compounds with transition metal ions (USEPA 1997). In addition, its related compounds, such as phenolic compounds, have been used in industries such as plywood, construction and appliance (Gallego et al. 2003), and are commonly detected in contaminated environments. Because the phenolic compounds and *p*-chlorocresol cause serious threats to human health, they are included in the list of 129 priority pollutants, according to the US Environmental Protection Agency (Keith 1979). The use of *p*-chlorocresol in cosmetics is restricted in Europe and Japan (Gallego et al. 2003).

A few studies on the degradation of *p*-chlorocresol have been published (Ziagova and Liakopoulou-Kyriakides 2007; Ziagova et al. 2009), but the anaerobic degradation of the

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-019-2025-9>) contains supplementary material, which is available to authorized users.

✉ Duc Danh Ha
hadanhduc@gmail.com

¹ Dong Thap University, Cao Lanh City, Dong Thap Province, Viet Nam

compound has not yet been reported in detail. The anaerobic degradation of aromatic compounds usually occurred under denitrifying, methanogenic, sulfate-reducing, and ferric iron-reducing conditions. The use of nitrate as an electron acceptor for anaerobic degradation is advantageous, because it is highly soluble in water, inexpensive, and has no toxicity.

Thus, the aims of this study were to (1) isolate and identify *p*-chlorocresol-degrading bacteria under both aerobic and anaerobic conditions, (2) investigate the biodegradability of *p*-chlorocresol and related compounds in liquid and sediment slurry media, (3) investigate oxygen effects on the degradation rates of *p*-chlorocresol, and (4) determine the degradation pathways for the compound.

Materials and methods

Chemicals and media

The mineral salts medium (MSM) components were K_2HPO_4 1.5 g/L, KH_2PO_4 0.5 g/L, $(\text{NH}_4)_2\text{SO}_4$ 1.0 g/L, NaCl 0.5 g/L, MgSO_4 0.2 g/L, CaSO_4 0.5 g/L and 1 mL of trace elements solution (39.9 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 42.8 mg $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$, 3.8 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 11.6 mg H_3BO_3 , 27.8 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 10 mg MoO_3 per liter) (Ha 2018). The pH of the liquid media was adjusted to 7.0 ± 0.1 . The medium was sterilized at 121°C for 15 min. Organic substrates were dissolved in absolute ethanol as stock solutions at 0.1 M. Solid medium was obtained by adding 2% (w/v) agar for cell cultivation. All chemicals were purchased from Sigma-Aldrich (USA) or Merck (Germany).

Isolation and identification of *p*-chlorocresol-degrading bacteria

Several aerobic and anaerobic sludge and sediment samples were collected from a site on the Mekong River ($10^\circ19'23\text{N}$, $115^\circ45'20\text{E}$, Dong Thap Province, Vietnam), where there are effluents of some industrial complexes and domestic wastewater. The samples were mixed and washed through a 1.0 mm, pore size mesh with site water. Samples were then diluted to 1/10th with MSM medium (w/v) and used for enrichment culture. 70 mL of the solution was transferred into a laboratory reactor. The reactor was supplemented with 0.3 mM *p*-chlorocresol and 10 mM nitrate (NaNO_3).

The laboratory reactor was fabricated using a glass vial connected to oxygen and nitrogen gas tanks through air controls (Fig. 1). Filtered sterile air was introduced from the vial bottom with a total flow rate of 5.0 mL min^{-1} . The aerobic and anaerobic conditions were created by blowing with 79% and 100% nitrogen gas, respectively. The vial was incubated for 1.0 week under the aerobic condition, followed by 1.0 week under the anaerobic condition at room temperature

Fig. 1 Flowsheet for the batch degradation of chemicals

(from 28.0 to 31.5°C). After 4 weeks, the solution was diluted and spread onto MSM agar plates supplemented with 0.3 mM *p*-chlorocresol. Isolated colonies were sub-cultured onto further agar plates to obtain pure strains. Each bacterial strain was transferred to MSM liquid medium to check the growth and biodegradation ability towards *p*-chlorocresol as a sole carbon and energy source. The identification of the obtained bacterial strain and construction of phylogenetic tree were described by Ha (2018).

Cell growth and biodegradation of *p*-chlorocresol and its derivatives in liquid media

The biodegradation experiments were carried out in the MSM medium with or without supplementation with glucose (1.0 g L^{-1}). The bacteria were cultured in liquid LB medium for 18 h, used for inoculation. The cell inoculum (0.5 mL) was transferred to the reactor to give an original cell number of approximately 10^6 CFU mL^{-1} (based on the plate count method). Individual chemical compounds (phenol, toluene, *m*-cresol, 4-chlorophenol, 2-chlorotoluene, and *p*-chlorocresol) were added at specific concentrations. Resazurin (0.4 mM) was used as an indicator to confirm the aerobic and anaerobic conditions. The reactor system was operated as described above. Samples (2.0 mL) were collected using syringes at specific time periods to determine chemical degradation, cell growth, metabolite products, and electron acceptor transformation.

Identification of metabolites and degradation pathways for *p*-chlorocresol

The metabolites were determined during *p*-chlorocresol degradation by resting cell culture. Bacteria were cultured in the MSM medium amended with glucose (1.0 g L^{-1}). After 12 h of incubation, cells were harvested by centrifuging for

5 min at 12,000 rpm. Cell pellets were resuspended in the MSM medium to give the final optical density (OD_{600}) of approximately 2.0. *p*-Chlorocresol (0.3 mM) was added to the medium, and the incubation processes were carried out under aerobic and anaerobic conditions.

Degradation intermediates were extracted with an equal volume of ethyl acetate three times. The solution was evaporated under a nitrogen stream and then dissolved in acetonitrile (HPLC grade). The metabolites from *p*-chlorocresol degradation were analyzed using a Finnigan-MAT MD8000 GC-MS (San Jose, CA, USA) equipped with a DB-5 capillary column (30 m $\times$ 0.5 mm $\times$ 0.25 μ m), and helium was used as the carrier with a flow of 1 mL min⁻¹. The applied oven temperature program was set up at 80 °C for 5 min, then raised 15 °C per min to 260 °C and maintained for 3 min at this temperature. The MS was equipped with an electron ionization source at the electron energy of 70 eV. The scanning intervals were 1.5 times s⁻¹, and the mass ranged from 30 to 220 m/z. Samples were injected into the GC-MS instrument in the split ratio of 1:5. The activities of enzymes involving the meta- and ortho-cleavage pathways in aerobic degradation were also assayed according to the previous report (Duc 2017). Catechol 1, 2-dioxygenase activity was assayed spectrophotometrically by measuring the formation of cis, cis-muconic acid of catechol at 260 nm, while catechol 2, 3-dioxygenase activity was assayed by following the formation of 2-hydroxymuconic semialdehyde at 375 nm. In addition, the determination of the enzymes produced in anaerobic degradation (3-hydroxybenzyl alcohol dehydrogenase, 3-hydroxybenzaldehyde dehydrogenase, 3-hydroxybenzoate CoA ligase, benzoate-CoA ligase, and benzoyl-CoA reductase) was described by Bonting et al. (1995).

Anaerobic degradation of *p*-chlorocresol and related compounds by bacteria in slurry sediment

Sediment samples collected from the Mekong River near the location described above were mixed and washed through a 1.0 mm, pore size mesh with site water. The indigenous bacteria emerging on solid LB agar under aerobic and anaerobic conditions were $2.6 \times 10^6 \pm 0.1 \times 10^6$ and $5.5 \times 10^4 \pm 0.3 \times 10^4$ CFU g⁻¹ dried sample, respectively. The experiment was carried out according to a previous report (Ha 2018), with some modification. The sediment was mixed with site water to gain a slurry with 10% dry matter (w/v) and pH 6.1. Then, 70 mL of the slurry was transferred into the serum glass vial. Nitrate and bacteria were added with the same concentrations as those in the liquid media, while individual chemicals were supplemented at 0.3 mM L⁻¹ slurry. The degradation conditions were the same as the degradation in liquid media. The dried sediment components were analyzed based on

Hossain et al. (2017) and are shown in Table 1. Samples were collected to determine the chemical remediation. The substrates were extracted, and the mean recovery of phenol, toluene, *m*-cresol, 4-chlorophenol, 2-chlorotoluene, and *p*-chlorocresol from the slurry was 92.4%, 97.8%, 96.4%, 97.5%, 95.4% and 96.6%, respectively.

Analytical methods

HPLC with a column from Waters (250 mm $\times$ 4.5 mm and 5 μ m particle size) was used to analyze the chemical concentrations. The mobile phase (acetonitrile/water: 75/25, v/v) was run at the flow rate of 1.0 mL min⁻¹. The UV-900 wavelength absorbance detector was used to monitor at 215 nm. The results were compared with the retention times and authentic standards of compounds with known concentrations to obtain qualitative and quantitative data. A DU800 spectrophotometer (Beckman Coulter Inc., Brea, CA, USA) was used to determine cell turbidity at 600 nm (OD_{600}). The exponential growth rate was determined based on the OD levels at the beginning and at specific times. The Lowry method was used to measure the concentrations of protein extracted from cells, in which bovine serum albumin served as the protein standard (Lowry et al. 1951).

Statistical analysis

The experiments were done at least in triplicate. Data were presented as means $\pm$ standard deviation. The SPSS software (version 22.0) was used to analyze variance, and significant differences ($p < 0.05$) were calculated using the Duncan's test.

Table 1 Components of the dry sediment

Composition	Units	Statistics
Clay	%	12.2
Silt	%	47.7
Sand	%	40.1
Total organic carbon	g kg ⁻¹	4.0
Total nitrogen	g kg ⁻¹	0.3
Si	g kg ⁻¹	301.2
Al	g kg ⁻¹	36.6
Ca	g kg ⁻¹	12.2
Fe	g kg ⁻¹	15.1
Mg	g kg ⁻¹	9.5
K	g kg ⁻¹	8.8
S	g kg ⁻¹	2.1
P	g kg ⁻¹	0.5
Mn	g kg ⁻¹	0.4

Results and discussion

Isolation and identification of *p*-chlorocresol-degrading bacterium

Several bacterial isolates could aerobically and anaerobically grow on *p*-chlorocresol as a sole carbon and energy source selected for the next experiments. Only bacterial strain DO utilizing a wide range of substituted aromatic compounds was selected for more detailed investigation. The 16S rRNA sequence is 1415 bp, which is closely related to the sequences of *Thauera* sp. available on the NCBI GenBank. The Ribosomal Database Project (<https://rdp.cme.msu.edu>) analysis also showed that the isolate DO belongs to the genus *Thauera*. The comparison of the obtained 16S rRNA gene to other sequences on BTASTn shows that the isolate KT was highly similar to *Thauera* sp. T1 (99% similarity), *Thauera aromatica* strain M11 (99% similarity), and other species shown in the phylogenetic tree (Fig. 2). Accordingly, the isolate was named *Thauera* sp. DO. The obtained 16S rDNA sequence has been deposited in GenBank under accession number MH681606.1, and the isolate has been deposited in the Culture Collection at the Center for Biochemical Analysis (Dong Thap University, Vietnam) under the number DUCOANH2017-DO.

Degradation of *p*-chlorocresol by *Thauera* sp. DO under aerobic and anaerobic conditions

The degradation rates and growth rates of bacteria in liquid media containing *p*-chlorocresol or *p*-chlorocresol-derived compounds were determined. The *p*-chlorocresol-derived compounds used in this study included substrates in which one or two substituents in the *p*-chlorocresol molecule are replaced by hydrogen atoms (Table 2). *Thauera* sp. DO showed a high efficiency of phenol and toluene degradation, while the isolate utilized *p*-chlorocresol with the lowest rates compared with other compounds tested in both aerobic and anaerobic conditions. Bacteria grew with higher rates on substrates with easier degradation in most treatments. The degradation rates of all substrates by the aerobic culture were comparably higher than those by the anaerobic culture (Table 2). No statistical reduction of any chemicals in abiotic controls was found (data were not shown).

The glucose addition increased the degradation of 4-chlorophenol, 2-chlorotoluene, and *p*-chlorocresol by 22.8%, 31.4%, and 35.5% under the aerobic condition, and 28.8%, 30.5%, and 31.4% under the anaerobic condition after 3 h, respectively. Even though *p*-chlorocresol degradation was lower than other substrates, only some research on *p*-chlorocresol biodegradation has been reported. Thus, the degradation ability and the degradation routes for this compound in *Thauera* sp. DO were further investigated.

The *p*-chlorocresol degradation by *Thauera* sp. DO was more effective than any of the microorganisms in previous

Fig. 2 Phylogenetic tree illustrating the evolutionary relationship between *Thauera* sp. DO (bold type) and other related species constructed based on the 16S rRNA gene sequences

Table 2 Exponential growth and utilization rates of phenols and toluenes as sources of carbon and energy by *Thauera aromatica* KT

Substrates	Aerobic degradation*		Anaerobic degradation*	
	Degradation rate ($\mu\text{M h}^{-1}$)	Bacterial growth rate (h^{-1})	Degradation rate ($\mu\text{M h}^{-1}$)	Bacterial growth rate (h^{-1})
Phenol	49.5 ± 9.3^A	0.060 ± 0.006^A	34.2 ± 5.5^A	0.033 ± 0.004^A
Toluene	47.5 ± 8.5^{AB}	0.066 ± 0.007^A	31.2 ± 3.9^{AB}	0.034 ± 0.004^A
<i>m</i> -Cresol	43.5 ± 5.7^{AB}	0.056 ± 0.007^A	26.8 ± 4.1^{BC}	0.025 ± 0.003^B
4-Chlorophenol	37.8 ± 5.6^{BC}	0.044 ± 0.006^B	20.8 ± 4.1^{CD}	0.022 ± 0.002^{BC}
2-Chlorotoluene	34.1 ± 3.2^{CD}	0.038 ± 0.005^{BC}	18.5 ± 2.3^D	0.019 ± 0.002^C
<i>p</i> -Chlorocresol	21.5 ± 2.7^D	0.030 ± 0.004^C	11.0 ± 1.2^E	0.012 ± 0.002^D

Bacteria were cultured in the MSM medium, supplemented with 0.3 mM individual substrates for 6 h

*Results are shown as means $\pm$ SD, in which different superscript letters (A, B, C, and D) denote significant difference ($p < 0.05$) among treatments in a column based on Duncan's test, whereas the same letter indicates no significant difference

reports. The aerobic *p*-chlorocresol degradation by *Pseudomonas* sp. (Ziagova and Liakopoulou-Kyriakides 2007) and *Staphylococcus xylosus* (Ziagova et al. 2009) required the glucose amendment into media. However, *Pseudomonas* sp. degraded only between 8 and 37% of this compound in a range of concentrations from 0.1 to 1.0 mM (Ziagova and Liakopoulou-Kyriakides 2007); *Staphylococcus xylosus* could not grow at concentrations higher than 0.5 mM in aerobic media (Ziagova et al. 2009). In another study, 5 and 10 ppm of the compound was degraded by the microbial inoculum in settled domestic wastewater after 14 days (Tabak et al. 1981). From 30 to 100% of *p*-chlorocresol was degraded after 3 weeks under aerobic conditions, but no degradation was detected in anaerobic media (Voets et al. 1976).

Some anaerobic pure cultures degrading phenol, cresols and toluene (Lovley and Lonergan 1990; Kar et al. 1997), and chlorophenols (Arora and Bae 2014; Li et al. 2014) have

been described. However, no anaerobic chlorotoluenes-degrading microbial strain has been reported. Moreover, only toluene-degrading *Thauera* sp. strain DNT-1 under both aerobic and anaerobic conditions was described by Shinoda et al. (2004).

Effects of *p*-chlorocresol concentrations on cell growth and biodegradation rates

To evaluate the effects of *p*-chlorocresol concentrations on cell growth and biodegradation, *Thauera* sp. DO was cultivated in the MSM medium supplemented with various *p*-chlorocresol concentrations under denitrifying conditions. The degradation rates of *p*-chlorocresol depended on the chemical concentrations (Fig. 3). Under the aerobic condition, *Thauera* sp. DO completely utilized 0.1 mM of the substrates within 24 h, and the increase of concentrations resulted in lower degradation

Fig. 3 Degradation of *p*-chlorocresol (a) and cell growth on the substrate (b) at 0.1 mM (filled diamond), 0.3 mM (filled square), 0.5 mM (filled triangle), 0.8 mM (filled circle) and 1.0 mM (unfilled circle) under

the aerobic condition. The degradation experiments were carried out at the room temperature and pH 7. The control without bacteria (unfilled circle) was run in parallel

rates. *Thauera* sp. DO could utilize *p*-chlorocresol up to 0.8 mM, and the strain was completely inhibited to grow at 1.0 mM in the medium without glucose.

In the anaerobic media, the degradation rates of any chemical and bacterial growth rates after 24 h were significantly lower than those under the aerobic condition ($p < 0.05$). KT completely degraded 0.1 mM *p*-chlorocresol within 30 h of incubation, but it could not grow at the concentrations higher than 0.3 mM under the anaerobic condition (Fig. 4). The anaerobic removal of the chemical in controls without bacteria or without the electron acceptor was negligible.

Stoichiometry between nitrate transformation and substrate degradation by the anaerobic culture

The half reaction for the complete anaerobic oxidation of phenol, toluene, *m*-cresol, 4-chlorophenol, 2-chlorotoluene and *p*-chlorocresol to CO_2 could be stated as the following equations of 1, 2, 3, 4, 5 and 6, respectively:

Nitrate might be transferred to nitrogen gas or/and nitrite as the following:

According to Eqs. 7 and 8, the amount of electrons consumed to reduce 1 M nitrate to nitrite and nitrogen gas were 2 and 5 mol, respectively. The electron balances of chemical consumption and nitrate reduction were obtained based on theoretical calculations and presented in Table 3. The amount of electrons consumed was lower than those expected for the complete chemical oxidation to CO_2 . This deviation was probably because partial substrates were used for cell synthesis, and the mineralization processes were not complete.

Effects of oxygen concentrations on degradation of *p*-chlorocresol by *Thauera* sp. DO

In natural environments such as contaminated aquifers, low-oxygen concentrations frequently occur due to the consumption by abiotic and biotic processes and low water solubility of oxygen, which affects the degradation of xenobiotics. In this study, the facultative *Thauera* sp. DO degraded *p*-chlorocresol in both aerobic and anaerobic media. The increase of oxygen concentrations from 0 to 40% enhanced the degradation rates. However, the increase of oxygen concentrations

Fig. 4 Degradation of *p*-chlorocresol (a) and cell growth on the substrate (b) at 0.1 mM (diamond), 0.2 mM (x), 0.3 mM (square) and 0.5 mM (triangle) under the anaerobic condition. The degradation experiments were carried out at room temperature and pH 7

Table 3 Electron balance and nitrate reduction during the anaerobic degradation processes carried out at the initial concentration of 0.3 mM of each substrate for 6 h

Substrates	Amount of substrate consumed (mmol)	Amount of electrons produced (mmol)	Amount of nitrate reduced to nitrite (mmol)	Amount of nitrate reduced to nitrogen gas (mmol)	Ratio of electrons consumed to produced (%)
Phenol	0.21 ± 0.03	5.74 ± 0.92	0.18 ± 0.04	0.99 ± 0.12	92.8 ± 6.3
Toluene	0.19 ± 0.02	6.73 ± 0.84	0.24 ± 0.04	1.14 ± 0.15	92.1 ± 3.2
<i>m</i> -Cresol	0.16 ± 0.02	5.47 ± 0.83	0.25 ± 0.04	0.83 ± 0.15	85.0 ± 5.0
4-Chlorophenol	0.12 ± 0.02	3.15 ± 0.58	0.20 ± 0.03	0.46 ± 0.09	86.2 ± 6.8
2-Chlorotoluene	0.11 ± 0.01	3.77 ± 0.47	0.28 ± 0.05	0.51 ± 0.08	82.9 ± 8.4
<i>p</i> -Chlorocresol	0.07 ± 0.01	2.12 ± 0.26	0.18 ± 0.03	0.27 ± 0.04	79.8 ± 5.5

Fig. 5 Effects of oxygen concentrations on the degradation of *p*-chlorocresol by *Thauera aromatica* KT. The degradation was carried out at the initial concentration of 0.3 mM, and the degradation rates were determined at the 6th h of incubation. The degradation experiments were carried out at room temperature and pH 7

from 42 to 96% did not statistically change the consumption rates (Fig. 5).

Degradation pathways for *p*-chlorocresol and related compounds in *Thauera* sp. DO

During the degradation of *p*-chlorocresol and other compounds mentioned above, the degradation products were isolated and identified by GC–MS. In the aerobic liquid media, an intermediate showed a molecular ion at m/z 108, 107, 79, 77, matching the authentic standard of *m*-cresol (Fig. S1). Catechol (m/z 110, 63, 64) (Fig. S2) and 4-chlorocatechol (m/z 144, 146, 63) (Fig. S3) were also detected during the aerobic degradation. Under the anaerobic condition, *m*-cresol also appeared as an intermediate. These results indicated that the dechlorination occurred in both the aerobic and anaerobic conditions.

The activities of enzymes extracted from bacteria cultured on different carbon sources versus the cells cultured on yeast extract are shown in Table 4. Under the aerobic condition, the specific activity of catechol 1, 2-dioxygenase was significant, while the activity of catechol-2, 3-dioxygenase was negligible. For anaerobic degradation, the activities of 3-hydroxybenzyl alcohol dehydrogenase, 3-hydroxybenzaldehyde dehydrogenase, 3-hydroxybenzoate CoA ligase, and

Table 4 Specific enzyme activities related to aerobic and anaerobic metabolism in extracts of *Thauera aromatica* KT grown on various carbon sources

Substrates	Enzyme activity [(μmol (min.mg protein) ⁻¹)]			
	Aerobic degradation		Anaerobic degradation	
	Catechol 1, 2-dioxygenase	Catechol-2, 3-dioxygenase	3-Hydroxybenzoate CoA ligase	Benzoate-CoA ligase
Phenol	1.07 ± 0.12	0.07 ± 0.01	1.84 ± 0.22	2.82 ± 0.31
Toluene	0.66 ± 0.07	< 0.01	1.07 ± 0.11	1.97 ± 0.14
<i>m</i> -Cresol	1.05 ± 0.11	0.06 ± 0.01	1.51 ± 0.17	2.11 ± 0.24
4-Chlorophenol	1.33 ± 0.14	0.04 ± 0.00	1.65 ± 0.12	2.67 ± 0.20
2-Chlorotoluene	0.55 ± 0.06	< 0.01	0.97 ± 0.11	2.06 ± 0.16
<i>p</i> -Chlorocresol	0.47 ± 0.05	0.05 ± 0.01	0.88 ± 0.10	1.78 ± 0.24
Yeast extract	0.23 ± 0.02	< 0.01	0.33 ± 0.00	0.86 ± 0.08

Bacteria were cultured in the MSM medium supplemented with 0.3 mM individual substrates for 6 h

benzoyl-CoA reductase were from 0.1 to 0.5 $\mu\text{mol (min.mg protein)}^{-1}$, which were lower than those of 3-hydroxybenzoate CoA ligase and benzoate-CoA ligase shown in Table 4. The activities of enzymes extracted from cells cultured in medium with *p*-chlorocresol and related compounds were significantly higher than those from bacteria cultured on yeast extract. On the basis of metabolite identification, the plausible aerobic and anaerobic biodegradation pathways for *p*-chlorocresol in *Thauera* sp. DO could be proposed in Fig. 6. The metabolites produced in the aerobic, anaerobic, and two-stage degradation processes are also presented in Fig. 7.

Under the aerobic condition, *p*-chlorocresol was dechlorinated in the first step to *m*-cresol, then the methyl group was oxidized to 3-hydroxybenzoate. Another

aerobic pathway, dechlorination, did not occur in the first step, and 4-chlorocatechol was formed, which was subsequently completely degraded. The determination of enzyme activities demonstrated that the aerobic ring fission by *Thauera* sp. DO occurred via the ortho-cleavage pathway. A previous report showed that only one metabolic product, identified as 3-methyl-4-oxo-pentanoic acid, was found during the aerobic *p*-chlorocresol degradation by *Staphylococcus xylosus* (Ziagova and Liakopoulou-Kyriakides 2007). Under the anaerobic condition, *p*-chlorocresol was also dehalogenated, followed by the methyl oxidation process. The methyl oxidation of *m*-cresol was reported in a previous report (Bonting et al. 1995).

The metabolites produced during the degradation of other substrates were also investigated. The results

Fig. 6 Degradation pathways for *p*-chlorocresol in *Thauera aromatica* KT under aerobic and anaerobic conditions. Two consecutive arrows show undetermined metabolites

Fig. 7 *p*-Chlorocresol degradation (solid line) and formation of intermediate products (dashed line) by whole cells of *Thauera aromatica* KT. The biodegradation was carried out under the aerobic condition (a), anaerobic condition (b), and anaerobic condition (2 h) followed

by aerobic condition (c). During the degradation of *p*-chlorocresol (square), *m*-cresol (circle) and 4-chlorocatechol (triangle) were formed as metabolites. The degradation experiments were carried out at room temperature and pH 7

indicated that the intermediates of *m*-cresol degradation in both conditions were the same as the pathways shown in Fig. 6. The methyl group in toluene and 2-chlorotoluene was converted to the carboxyl group and further metabolized by both aerobic and anaerobic cultures of *Thauera* sp. DO. Phenol was transformed to catechol and 4-hydroxybenzoate under aerobic and anaerobic conditions, respectively. 4-Chlorophenol was dechlorinated to phenol in anaerobic media, while the compound was transformed to 4-chlorocatechol, which was completely mineralized by the aerobic culture.

Two-stage process of *p*-chlorocresol anaerobic-aerobic degradation

In media with high cell density (resting cells), the increase in cell numbers during the test was not statistically observed (Schmidt et al. 1985), which presents an immediate response of the biodegradation of a target compound by bacteria. In the aerobic medium, 0.3 mM *p*-chlorocresol was completely degraded by the whole cells within 12 h (Fig. 7a). In the anaerobic medium, around 40% of *p*-chlorocresol was dechlorinated to *m*-cresol within 2 h at the beginning,

and both the substrate and its product were then gradually reduced in the ensuing time (Fig. 7b). The low degradation rates of *p*-chlorocresol in the ensuing time were probably due to the inhibition of intermediates accumulated in the medium. To enhance the degradation, the anaerobic degradation was first carried out, followed by the stage of aerobic incubation (Fig. 7c). Because an amount of *p*-chlorocresol was dehalogenated, 4-chlorocatechol concentrations accumulated in the two-stage degradation process were lower than the concentrations accumulated in a single aerobic process. *m*-Cresol that appeared in the anaerobic transformation was rapidly degraded in the aerobic process (Fig. 7c).

Armenante et al. (1999) reported that using the two-stage anaerobic–aerobic process resulted in higher degradation rates of chlorinated phenols than any single-stage process. In the combination of these processes, the dechlorination that occurred by the anaerobic culture, and the complete degradation of its products was conducted in the aerobic condition (Armenante et al. 1999). The aerobic degradation was carried out after the anaerobic process, resulting in the complete degradation of pentachlorophenol, which was mostly dechlorinated but was not completely removed in anaerobic media (Wilson et al. 1997). The enhancement of the degradation ability towards 2, 4-dinitrotoluene by the application of the two-stage process was reported by Berchold et al. (1996).

Degradation of *p*-chlorocresol and related compounds by *Thauera* sp. DO in sediment slurry

Table 5 shows that the degradation rates of any substrate in the aerobic sediment slurry were significantly higher than those in the anaerobic ones. A small amount of substrates was absorbed into the sediment. The degradation in the anaerobic sediment slurry without nitrate addition was also carried out, and the results showed that the degradation rates of any substrate were reduced by approximately 45–55%, which indicated that the electron acceptor played an important role in anaerobic degradation. However, the degradation also occurred in spite of no nitrate supplementation, which showed that some components in the sediment could serve as electron acceptors.

The bioremediation rates in sediment slurry were significantly lower than the rates in liquid media, which was probably because the bacterial activities in the sediment were influenced by its physicochemical properties such as available nutrients, pH and other environmental factors. Moreover, the sorption of chemicals or/and microorganisms to sediments may reduce the availability to microorganisms. The amount of substrates dissipated in sterile slurry and non-sterile slurry was not statistically different, which indicated that the activities of native microorganisms were negligible, and the degradation by *Thauera* sp. DO was not affected by indigenous bacteria.

A number of publications have described the microorganism's bioaugmentation in liquid and soils. However, only a few studies on the augmentation for anaerobic degradation of aromatic compounds in sediments have been published

Table 5 Degradation of *p*-chlorocresol and related compounds by *Thauera aromatica* KT in sediment slurry

Conditions	Substrates	Degradation rate ($\mu\text{M h}^{-1}$)*			
		Non-inoculation		Inoculation with <i>Thauera aromatica</i> KT	
		Sterile slurry	Non-sterile slurry	Sterile slurry	Non-sterile slurry
Aerobic incubation	Phenol	$4.1 \pm 1.2^{\text{Ab}}$	$5.1 \pm 1.4^{\text{Ab}}$	$41.8 \pm 5.5^{\text{Aa}}$	$43.1 \pm 5.6^{\text{Aa}}$
	Toluene	$2.9 \pm 0.8^{\text{ABb}}$	$3.2 \pm 1.4^{\text{Cb}}$	$34.6 \pm 4.3^{\text{Ba}}$	$36.2 \pm 3.8^{\text{Ba}}$
	<i>m</i> -Cresol	$2.9 \pm 0.7^{\text{ABb}}$	$3.2 \pm 0.4^{\text{Cb}}$	$35.0 \pm 3.9^{\text{Ba}}$	$35.7 \pm 4.0^{\text{Ba}}$
	4-Chlorophenol	$3.0 \pm 0.3^{\text{ABb}}$	$4.0 \pm 0.3^{\text{ABCb}}$	$29.7 \pm 3.3^{\text{Ba}}$	$32.4 \pm 3.5^{\text{Ba}}$
	2-Chlorotoluene	$3.0 \pm 0.6^{\text{ABb}}$	$3.7 \pm 0.6^{\text{ABCb}}$	$29.4 \pm 3.1^{\text{Ba}}$	$32.0 \pm 3.6^{\text{Ba}}$
	<i>p</i> -Chlorocresol	$2.4 \pm 0.8^{\text{Bb}}$	$3.7 \pm 0.7^{\text{ABCab}}$	$16.5 \pm 2.2^{\text{Da}}$	$18.8 \pm 2.6^{\text{CDa}}$
Anaerobic incubation	Phenol	$4.1 \pm 0.7^{\text{Ab}}$	$4.8 \pm 0.6^{\text{ABb}}$	$29.4 \pm 3.1^{\text{Ba}}$	$32.1 \pm 3.4^{\text{Ba}}$
	Toluene	$2.4 \pm 0.1^{\text{Bb}}$	$2.8 \pm 0.5^{\text{Cb}}$	$23.0 \pm 2.0^{\text{Ca}}$	$24.4 \pm 1.5^{\text{Ca}}$
	<i>m</i> -Cresol	$2.4 \pm 0.4^{\text{Bb}}$	$3.1 \pm 0.8^{\text{Cb}}$	$22.7 \pm 2.0^{\text{Ca}}$	$23.7 \pm 2.0^{\text{CDa}}$
	4-Chlorophenol	$2.3 \pm 0.4^{\text{Bb}}$	$2.9 \pm 0.8^{\text{Cb}}$	$16.1 \pm 2.2^{\text{Da}}$	$18.5 \pm 2.7^{\text{Da}}$
	2-Chlorotoluene	$2.9 \pm 0.8^{\text{ABb}}$	$3.9 \pm 0.3^{\text{ABCb}}$	$15.3 \pm 1.2^{\text{Da}}$	$18.0 \pm 1.0^{\text{Da}}$
	<i>p</i> -Chlorocresol	$3.0 \pm 0.1^{\text{ABb}}$	$3.4 \pm 0.5^{\text{BCb}}$	$9.4 \pm 1.5^{\text{Ea}}$	$10.3 \pm 1.2^{\text{Ea}}$

The degradation rates were determined 6 h after incubation, and chemicals were supplemented with the initial concentration of 0.3 mM

*Results are shown as means $\pm$ SD, in which different superscript (A, B, C and D) and small letters (a and b) indicate statistically significant differences ($p < 0.05$) among treatments in the same group within a column and a line, respectively

(Ahn et al. 2008; Payne et al. 2011, 2013). The degradation determination in this study partly elucidated the effects of complicated conditions in the sediment on bioremediation, which supports the possibility of using the isolate KT for in situ bioaugmentation.

Conclusion

To our knowledge, *Thauera* sp. DO is the first to report that the strain utilized *p*-chlorocresol and some related compounds as a sole carbon and energy source under both aerobic and anaerobic conditions. The degradation rates of *p*-chlorocresol and degradation pathways depended on oxygen concentrations. This work also presents a novel method to increase the degradation rates using a two-stage process of anaerobic–aerobic degradation.

Acknowledgements This work was supported by Dong Thap University for research groups. The authors are very thankful for all funds to carry out this research work.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Acharya P, Ives P (1994) Incineration at Bayou Bounfouca remediation project. *Waste Manag* 14:13–26
- Ahn Y-B, Liu F, Fennell DE, Häggblom MM (2008) Biostimulation and bioaugmentation to enhance dechlorination of polychlorinated dibenzo-*p*-dioxins in contaminated sediments. *FEMS Microbiol Ecol* 66:271–281
- Andersen A (2006) Final report on the safety assessment of sodium *p*-chloro-*m*-cresol, *p*-chloro-*m*-cresol, chlorothymol, mixed cresols, *m*-cresol, *o*-cresol, *p*-cresol, isopropyl cresols, thymol, *o*-cymen-5-ol, and carvacrol. *Int J Toxicol* 25(Suppl. 1):129–127
- Armenante PM, Kafkewitz D, Lewandowski GA, Jou C-J (1999) Anaerobic–aerobic treatment of halogenated phenolic compounds. *Water Res* 33:681–692
- Arora PK, Bae H (2014) Bacterial degradation of chlorophenols and their derivatives. *Microb Cell Fact* 13:31
- Berchold SR, van der Loop SL, Suidan MT, Maloney SW (1996) Treatment of 2, 4-dinitrotoluene using a two-stage system: fluidized-bed anaerobic granular activated carbon reactors and aerobic sludge reactors. *Water Environ Res* 67:1081–1091
- Bonting CFC, Schneider S, Schmidtberg G, Fuchs G (1995) Anaerobic degradation of *m*-cresol via methyl oxidation to 3-hydroxybenzoate by a denitrifying bacterium. *Arch Microbiol* 164:63–69
- Buisson RSK, Kirk PWW, Lester JN (1984) Determination of chlorinated phenols in water, wastewater, and wastewater sludge by capillary GC/ECD. *J Chromatogr Sci* 22:339–342
- Dooms-Goossens A, Degreef H, Vanhee J, Kerkhofs L, Chrispeels MT (1981) Chlorocresol and chloracetamide: allergens in medications, glues, and cosmetics. *Contact Dermat* 7:51–52
- Duc HD (2017) Degradation of chlorotoluenes by *Comamonas testoteronae* KT5. *Appl Biol Chem* 60:457–465
- Gallego A, Fortunato MS, Foglia J, Rossi S, Gemini V, Gomez L, Gomez CE, Higa LE, Korol SE (2003) Biodegradation and detoxification of phenolic compounds by pure and mixed indigenous cultures in aerobic reactors. *Int Biodeterior Biodegrad* 52:261–267
- Great Lakes Water Quality Board (1983) An inventory of chemical substances identified in the great lakes ecosystem. Volume 1—summary. Report to the great lakes water quality board, Windsor Ontario, Canada
- Ha DD (2018) Anaerobic degradation of 2, 4-dichlorophenoxyacetic acid by *Thauera* sp. DKT. *Biodegradation* 29:499–510
- Hossain HMZ, Kawahata H, Roser BP, Sampei Y, Manaka T, Otani S (2017) Geochemical characteristics of modern river sediments in Myanmar and Thailand: implications for provenance and weathering. *Chem Erde Geochem* 77:443–458
- Huang Z, Zhou W, Zhou C, Han S, Zhang A, Feng F, Liu C, Wang L (2003) Determination of semivolatile compounds in drinking water by tandem mass spectrometric detection. *Bull Environ Contam Toxicol* 71:1026–1033
- James RH, Adams RE, Finkel JM, Miler HC, Johnson LD (1985) Evaluation of analytical methods for the determination of POHC (principal organic hazardous constituents) in combustion products. *Air Pollut Control Assoc* 35:959–969
- Kar S, Swaminathan T, Baradarajan A (1997) Biodegradation of phenol and cresol isomer mixtures by *Arthrobacter*. *World J Microbiol Biotechnol* 13:659–663
- Keith LH (1979) Priority pollutants I—a perspective view. *Environ Sci Technol* 13:416–423
- Kool HJ, van Kreijl CF, Zoeteman BCJ, Tardiff RG (1982) Toxicology assessment of organic compounds in drinking water. *C R C Crit Rev Environ Sci Technol* 12:307–357
- Kot-Wasik A, Kartanowicz R, Dabrowski D, Namieśnik J (2004) Determination of chlorophenols and phenoxyacid herbicides in the Gulf of Gdansk, southern Baltic Sea. *Bull Environ Contam Toxicol* 73:511–518
- Lewis RJ Sr (2007) Hawley's condensed chemical dictionary, 15th edn. John Wiley & Sons Inc., New York, p 280
- Li Z, Suzuki D, Zhang C, Yang S, Nan J, Yoshida N, Wang A, Katayama A (2014) Anaerobic 4-chlorophenol mineralization in an enriched culture under iron-reducing conditions. *J Biosci Bioeng* 118:529–532
- Lopes TJ, Furlong ET (2001) Occurrence and potential adverse effects of semivolatile organic compounds in streambed sediment, United States, 1992–1995. *Environ Toxicol Chem* 20:727–737
- Lovley DR, Lonergan DJ (1990) Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. *Appl Environ Microbiol* 56:1858–1864
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Bio Chem* 193:265–275
- Maibach HI (2001) Toxicology of the skin. Br J Dermatol, New York, Philadelphia: Taylor and Francis Inc. ISBN 1560328029
- Payne RB, Fagervold SK, May HD, Sowers KR (2013) Remediation of polychlorinated biphenyl impacted sediment by concurrent bioaugmentation with anaerobic halo-respiring and aerobic degrading bacteria. *Environ Sci Technol* 47:3807–3815
- Payne RB, May HD, Sowers KR (2011) Enhanced reductive dechlorination of polychlorinated biphenyl impacted sediment by bioaugmentation with a dehalorespiring bacterium. *Environ Sci Technol* 45:8772–8779
- Riemer C, Bamme T, Mok SW, Baier M (2006) 3-Methyl-4-chlorophenol for prion decontamination of medical devices. *Infect Control Hosp Epidemiol* 27:778–780
- Schmidt SK, Simkins S, Alexander M (1985) Models for the kinetics of biodegradation of organic compounds not supporting growth. *Appl Environ Microbiol* 50:323–331
- Schnaak W, Küchler T, Kujawa M, Henschel KP, Süßenbach D, Donau R (1997) Organic contaminants in sewage sludge and their

- ecotoxicological significance in the agricultural utilization of sewage sludge. *Chemosphere* 35:5–11
- Shinoda Y, Sakai Y, Uenishi H, Uchihashi Y, Hiraishi A, Yukawa H, Yurimoto H, Kato N (2004) Aerobic and anaerobic toluene degradation by a newly isolated denitrifying bacterium, *Thauera* sp. strain DNT-1. *Appl Environ Microbiol* 70:1385–1392
- Stangroom SJ, Collins CD, Lester JN (1998) Sources of organic micro-pollutants to lowland rivers. *Environ Technol* 19:643–666
- Tabak HH, Quave SA, Mashni CI, Barth EF (1981) Biodegradability studies with organic priority pollutant compounds. *J Water Pollut Control Fed* 53:1503–1518
- USEPA (1997) Office of pesticide programs; reregistration eligibility decision document—*p*-chloro-*m*-cresol, EPA 738-R-96-008
- Verschueren K (2001) Handbook of environmental data on organic chemicals, 4th edn. Wiley, New York
- Voets JP, Pipyn P, Van Lancker P, Verstraete W (1976) Degradation of microbicides under different environmental conditions. *J Appl Bacteriol* 40:67–72
- Wilson GJ, Khodadoust AP, Suidan MT, Brenner RC (1997) Anaerobic/aerobic biodegradation of pentachlorophenol using GAC fluidized bed reactors: optimization of the empty bed contact time. *Water Sci Technol* 36:107–115
- Ziagova M, Kyriakou G, Liakopoulou-Kyriakides M (2009) Co-metabolism of 2, 4-dichlorophenol and 4-Cl-*m*-cresol in the presence of glucose as an easily assimilated carbon source by *Staphylococcus xylosus*. *J Hazard Mater* 163:383–390
- Ziagova M, Liakopoulou-Kyriakides M (2007) Kinetics of 2, 4-dichlorophenol and 4-Cl-*m*-cresol degradation by *Pseudomonas* sp. cultures in the presence of glucose. *Chemosphere* 68:921–927