

**DEGRADATION OF CHLOROBENZENE AND 2-CHLOROTOLUENE
BY IMMOBILIZED BACTERIA STRAINS *Comamonas testosterone* KT5
AND *Bacillus subtilis* DKT**

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ABSTRACT

Chlorobenzenes and chlorotoluenes have been used to produce a number of industrial products. They are toxic and widely detected in environments due to human contributory negligence. In this article, the mixed culture of a toluenes-degrading bacterial strain, *Comamonas testosterone* KT5 (a Gram-positive, catalase-positive bacterium) and a chlorobenzenes-degrading bacterial strain, *Bacillus subtilis* DKT (a Gram-negative soil bacterium) effectively degraded both chemical compounds co-contaminating in liquid media. In addition, the degradations of mixed compounds by biofilm, bacteria immobilized in polyurethane foam (PUF) and alginate were determined. The results showed that the degradation of both compounds by cells in alginate was significantly higher than that by suspended cells. Moreover, cells immobilized in these materials showed lower adverse effects than those of non-immobilized cells for long-term storage. For examples, the degradation rates for chlorobenzene and 2-chlorotoluene by resting cells reduced by 39.5% and 37.3% after storage for 4 months at 4°C, while the degradation rates by immobilized cells decreased by from 16.3% to 19.8% respectively.

Keywords: *Bacillus subtilis* DKT, *Comamonas testosterone* KT5, chlorobenzenes, chlorotoluenes, degradation, immobilized cells.

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INTRODUCTION

Chlorobenzenes and chlorotoluenes are aromatic components widely used to produce a number of industrial products. For examples, chlorobenzenes are used to synthesize solvent, pesticides, dyes, pharmaceuticals, rubbers, plastics and disinfectants (Yadav et al., 1995; Zhang et al., 2005). Chlorotoluenes are components of agro-chemicals, flame retardants, dyes, pesticides, varnish and pigments, textile additives, pharmaceuticals, adhesives, polymers and resins, drain cleaners (Dobslaw & Engesser 2012). Meanwhile, they harmfully affect people and other species in food chains. Hence, these compounds are identified as priority pollutants by the U.S. Environmental Protection Agency (Zhang et al., 2011; Oh & Bartha, 1994).

Both chlorobenzenes and chlorotoluenes are commonly detected and persistent in environments. They are main components detected in river water, lake water, seawater and treated wastewater in Greece (Nikolaou et al., 2002). In other investigations, benzenes and toluenes co-contaminate water bodies (Slaine & Barker 1990; Nikolaou et al., 2002; Groschen et al., 2004; Mottaleb et al., 2003; Robinson et al., 2004; Waddell et al., 2004).

Biodegradation is the main mechanism for the removal of organic aromatic compounds contaminated environments because this method is an effectively, ecofriendly and cheap approach. A number of reports indicate that toluene and benzene inhibit to other during biodegradation processes (Chang et al., 1993; Oh et al., 1994; Bielefeldt & Stensel 1999; Reardon et al., 2000; Lin et al., 2007). In a previous report, the use of dual-species biofilm of *Comamonas testosterone* KT5 and *Bacillus subtilis* DKT could overcome this inhibition (Nguyen & Ha 2019). *Comamonas testosterone* KT5 effectively degraded some toluene compounds (Duc 2017), while *B. subtilis* DKT degraded a wide range of chlorobenzenes (Nguyen & Ha 2019). The dual-species biofilm of these isolates enhanced the degradation of both chlorobenzene and 2-chlorotoluene (Nguyen & Ha 2019).

For degradation of toxic substrates by microorganisms, cell immobilization is a preferred method because it may enhance degradation rates, reduces cell leakage and reactor volume. Some common immobilization methods such as biofilm, using alginate and PUF have been widely applied. This study describes the degradation of monochlorobenzene and 2-chlorotoluene by immobilized mixed-species of *C. testosterone* KT5 and *B. subtilis* BKT.

MATERIALS AND METHODS

Culture media

The mineral medium (MM medium) components were described by Duc (2017) supplemented with 0.1% yeast extract (w/v, MMY medium). The media were autoclaved at 121°C for 15 min. Monochlorobenzene and 2-chlorotoluene (purity > 99.5%) were purchased from Sigma–Aldrich (Singapore). These compounds were dissolved in methanol as stock solutions (0.1 M) prior to use.

Immobilization methods

Comamonas testosterone KT5 and *Bacillus subtilis* BKT were cultured in the MMY medium supplemented with 0.25 mM monochlorobenzene and 0.5 mM 2-chlorotoluene for 24 hours. The incubation was carried out at room temperature (30°C) and 150 rpm. Cells were harvested by centrifuging at 8,000 rpm for 5 min at 4°C. The cell pellets were then washed twice with saline (0.85% NaCl) and resuspended in MMY medium (resting cell culture, 5×10^{11} CFU/ml) and used for immobilization.

The alginate entrapment of bacteria was performed according to a previous study (Ha Danh Duc & Bui Minh Triet 2017). Bacterial cell suspension of the mixed culture was added to a sterilized alginate solution and mixed by stirring with a magnetic stirrer to give a final alginate concentration of 3%. This mixture was dropped using a syringe to a cold sterile CaCl₂ (0.2 M) solution to obtain beads with 3 mm in diameter. The cell numbers with approximately 2×10^8 CFUs/bead were yielded. The beads were washed twice with

saline and then hardened by submerging in a fresh sterile CaCl₂ solution for 8 hours in a static condition.

The PUF immobilization was carried out by the method described by Ha Danh Duc & Bui Minh Triet (2017) with modification. The PUF was cut into 5 mm cubes, placed in the packed reactor up to 70 cm³. 130 mL of resting cell culture were added to the reactor. The reactor was operated for 1 day allowing bacteria to immobilize in the PUF cubes.

For cell immobilization in ceramic rings, the carriers made from Jiangxi Yiyuan Industrial Co., Ltd, China were used. Each ceramic ring has a height × outer diameter of 1.5 × 1.5 cm, specific surface of 330 m²/m³, void volume of 0.7 m³/m³, bulk density of 690 kg/m³. Ceramic rings were placed in the packed reactor up to 70 cm³. 130 mL of resting cell culture were added into the reactor. The reactor was operated for 1 day for biofilm formation.

Set up and operational conditions of a packed reactor

A cylindrical, lab-scale packed reactor was set up using a glass column with the schematic illustrated in fig. 1. All reactor parameters were shown in table 1. The feed was pumped into the reactor on the top, while filter sterile air was introduced to the bottom through a ceramic diffuser. The packed reactor was operated at room temperature (30°C) in batch reactor. After 24 hours for each cycle, the spent medium was removed and the carriers were clean with sterile water twice. A new sterile MMY medium was then added for the next cycle. The chemical degradation was carried out by immobilized and resting cell culture of mixed species of *C. testosterone* KT5 and *B. subtilis* BKT. MMY medium with chlorobenzene (0.5 mM) and 2-chlorotoluene (1.0 mM) was used in all experiments.

Table 1. Main features of the packed reactor.

Attribute	Packed reactor
Height	15 cm
Diameter	5.5 cm (inside)
Liquid volume	130 cm ³
Aeration	0.75 mL air/mL liquid medium/min (Habibi & Vahabzadeh 2013)
Packed volume	70.0 cm ³



Figure 1. Ceramic rings used for cell immobilization

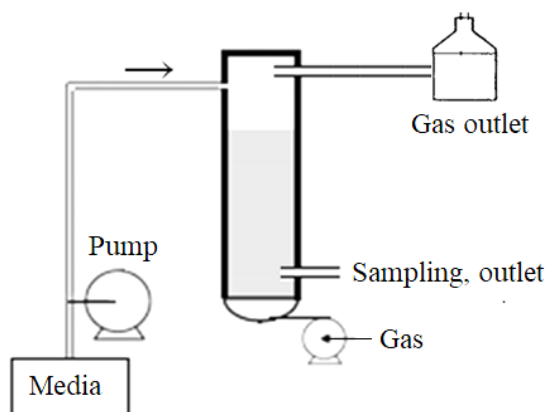


Figure 2. Schematic diagram of the lab-scale packed reactor

Storage conditions

The immobilized cells were used to degrade chlorobenzene and 2-chlorotoluene for 3 cycles in the reactor when cells on the carriers were saturated. These carriers with immobilized cells and resting cells were stored at 4°C and room temperature in a dark condition for 4 months.

Analytical method

Chlorobenzene and 2-chlorotoluene were measured using reverse phase high performance liquid chromatography (HPLC) (LC-10AD, Shimadzu, Japan) with a C18 column (5 µm, 250 mm×4.6 mm; Hyperclone, Phenomenex, USA) at absorbance of 240 nm. A mixture of acetonitrile and ultrapure water (7: 3, v/v) served as a mobile phase at a flow rate of 1 mL/min.

Cell numbers of each strain were determined by counting CFUs on solid MMY medium after 24 hours of incubation. *C. testosterone* KT5 formed round and convex colonies, while colonies of *B. subtilis* DKT were irregular, large size and undulate margin.

Statistical analysis

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

RESULTS AND DISCUSSION

Chemical degradation by mixed culture of *Comamonas testosterone* KT5 and *Bacillus subtilis* DKT in a packed reactor

The degradation of chlorobenzene and 2-chlorotoluene by resting cell culture and cells immobilized in alginate were compared. The results showed that the degradation percentages of chlorobenzene by immobilized cells and suspended cells for 24 hours were 84.4 ± 5.4 and $60.2\% \pm 7.1\%$, while data for 2-chlorotoluene were 92.2 ± 2.3 and $68.0 \pm 6.1\%$ in spite of the equal cell numbers (10^9 CFUs/ml), respectively. The alginate layer protected immobilized bacteria from chemical toxicity resulting in higher degradation compared to counterparts.

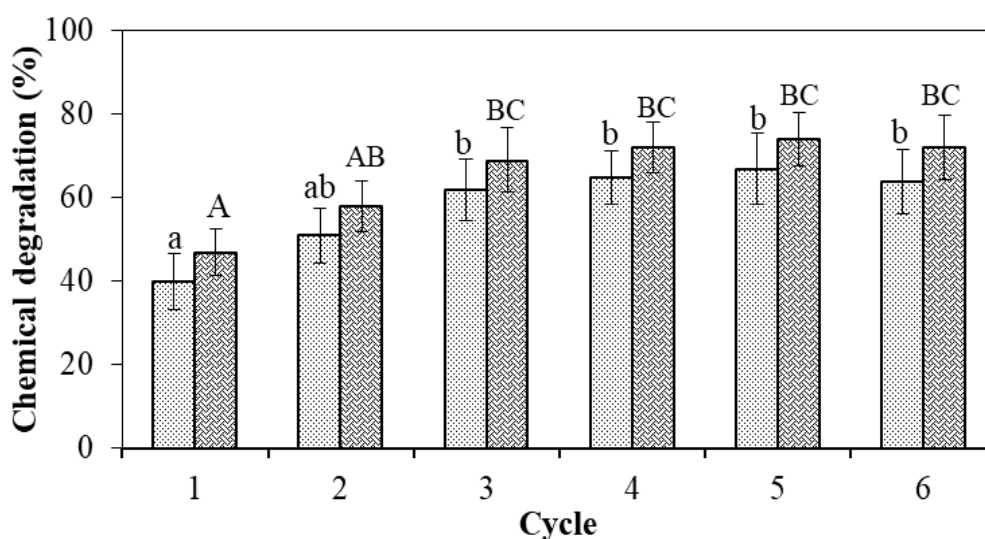


Figure 3. Degradation of chlorobenzene (□) and 2-chlorotoluene (▨) by mixed-species biofilm of *C. testosterone* KT5 and *B. subtilis* DKT on ceramic rings. Chlorobenzene and 2-chlorotoluene were used at 0.5 and 1.0 mM, respectively. Different small letters (a, b, c) indicate statistically significant differences ($p < 0.05$) in chlorobenzene degradation, while capital letters (A, B, C) indicate statistically significant differences in 2-chlorotoluene degradation

The degradation rates of chlorobenzene and 2-chlorotoluene by several types of immobilized cell mixture were compared. In our previous report, *B. subtilis* DKT synergistically promoted *C. testosteroni* KT5 to develop biofilm on carriers (Nguyen & Ha 2019). The degradation performances of both compounds by biofilm on ceramic rings were low in the first cycle (Fig. 3) probably due to low biofilm formation. However, the degradation increased in following cycles because more cells attached to the carrier. The degradation rates were not statistically different at the 3rd, 4th, 5th and 6th cycle (Fig. 3) probably because cell numbers in biofilm forming on the carriers did not change. The reductions of available O₂ and nutrients in inner biofilm

layer were the reasons that limit attached cell numbers (Lazarova & Manem, 1995).

Similar to the degradation by cell immobilized on ceramic rings, the degradation of chlorobenzene and 2-chlorotoluene by mixed-species culture in PUF was low at the first cycle (Fig. 4). Meanwhile, the degradation rates in other cycles were not statistically different. The degradation rates of these substrates by cell immobilized in PUF were higher than those in ceramic rings about 20% though both carriers had the same volume. The high porosity of PUF probably accounted for higher cell numbers immobilized in this material.

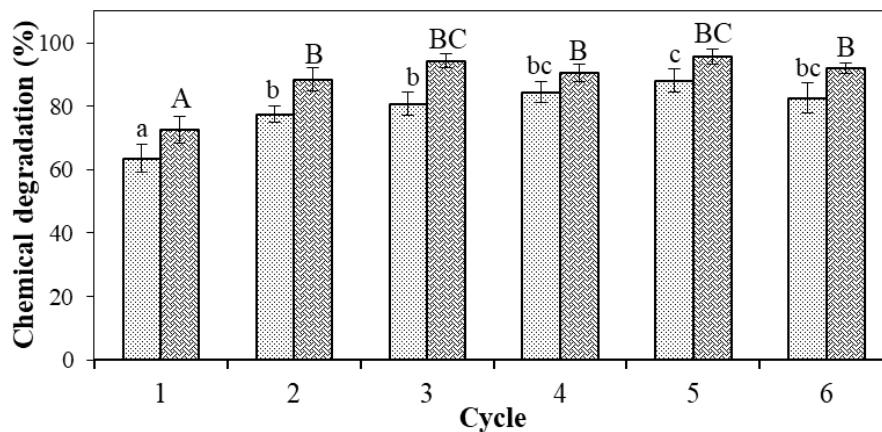


Figure 4. Degradation of chlorobenzene (□) and 2-chlorotoluene (■) by mixed-species of *C. testosteroni* KT5 and *B. subtilis* DKT immobilized in PUF. Chlorobenzene and 2-chlorotoluene were used at 0.5 and 1.0 mM, respectively. Different small letters (a, b, c) indicate statistically significant differences ($p < 0.05$) in chlorobenzene degradation, while capital letters (A, B, C) indicate statistically significant differences in 2-chlorotoluene degradation

For degradation by bacteria immobilized in alginate, the degradation rates were similar to those by bacteria immobilized in PUF. However, the rates were reduced in two last cycles (Fig. 5). The decrease of degradation occurred because some beads were broken and/or dissolved in solution resulting higher cell leakage (Table 1). The poor instability of alginate beads was also reported in a previous study (Ha Danh Duc & Bui Minh Triet, 2017).

During operation processes, bacteria detached from biofilm on ceramic rings, moved from PUF and alginate beads into liquid medium. The determination of cell numbers in liquid media with immobilization carriers from the first to third cycles were: ceramic rings \approx CFU $>$ alginate, at the fourth cycle were: ceramic rings \approx CFU \approx alginate, and at the fifth and sixth cycles were: ceramic rings \approx CFU $<$ alginate. Bacterial cell

numbers in liquid medium with ceramic rings and PUF were similar in all cycles, while those with alginate increased from the first to the 6th cycle (table 2). The alginate layer reduced bacteria to move into liquid media in early cycles. The determination of cell

numbers in the mixture in liquid media showed that cell numbers of each strain were similar (data were not shown). The previous study was also showed that both strains did not inhibit to other during the incubation (Nguyen & Ha 2019).

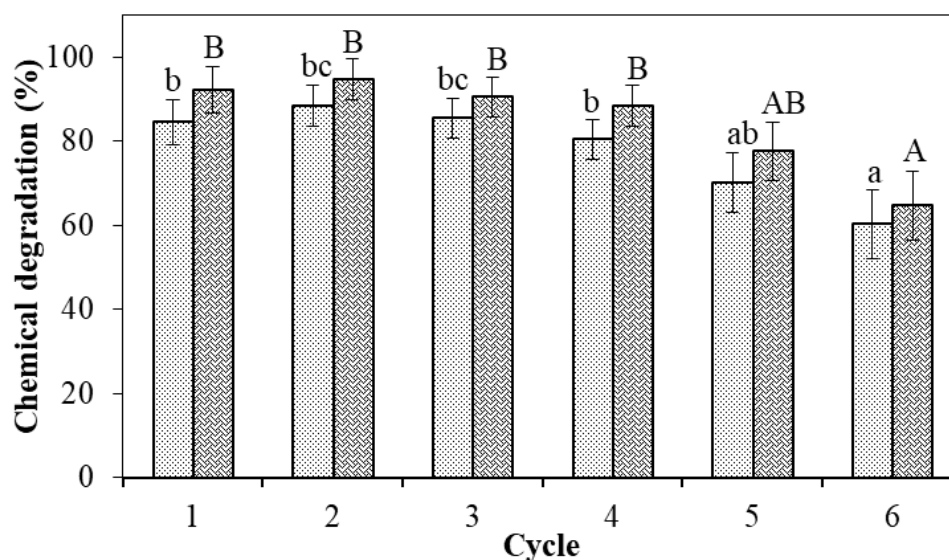


Figure 5. Degradation of chlorobenzene (▨) and 2-chlorotoluene (▩) by mixed-species of *C. testosterone* KT5 and *B. subtilis* DKT immobilized in alginate. Chlorobenzene and 2-chlorotoluene were added at 0.5 and 1.0 mM, respectively. Different small letters (a, b, c) indicate statistically significant differences in chlorobenzene degradation, while capital letters (A, B, C) indicate statistically significant differences in 2-chlorotoluene degradation ($p < 0.05$)

Table 2. Bacteria in liquid media in the reactor after 24 hours of operation.

Materials	Cell in liquid media ($\times 10^8$ CFUs/mL)*					
	1 st cycle	2 nd cycle	3 rd cycle	4 th cycle	5 th cycle	6 th cycle
Ceramic rings	0.72 ± 0.10^{aA}	0.78 ± 0.17^{aA}	0.81 ± 0.16^{aA}	0.83 ± 0.16^{aA}	0.80 ± 0.16^{aA}	0.84 ± 0.16^{aA}
PUF	0.71 ± 0.09^{aA}	0.71 ± 0.11^{aA}	0.74 ± 0.12^{aA}	0.75 ± 0.14^{aA}	0.73 ± 0.13^{aA}	0.69 ± 0.12^{aA}
Alginate	0.41 ± 0.05^{bA}	0.48 ± 0.03^{bA}	0.60 ± 0.06^{bAB}	0.74 ± 0.12^{aA}	1.14 ± 0.15^{bC}	1.25 ± 0.17^{bC}

Note: *: Different small letters (a and b) indicate statistically significant differences ($p < 0.05$) in the same columns, while capital letters (A, B and C) indicate statistically significant differences in the same lines.

Storage stability of free cells and immobilized bacterial cells in degrading chlorobenzene and 2-chlorotoluene

After 4-month storage at room temperature and 4°C, the biodegradation percentages of both chlorobenzene and 2-chlorotoluene were determined and shown in Figure 6. The decrease of degradation by immobilized cells was lower than resting cells. This result indicated that the

immobilized cells showed lower reduction of the adverse effects compared to free cells.

For storage at room temperature, the degradation rates of these compounds by resting cells reduced by 47.7% and 50.5%, respectively. The degradation rates of both compounds by immobilized cells decreased by from 28.1 to 33.3%, and these reductions of immobilized cells on each carrier were similar.

For storage at 4°C, the degradation rates of chlorobenzene and 2-chlorotoluene by resting cells reduced by 39.5 and 37.3%, respectively. The degradation rates of

immobilized cells decreased by from 16.3 to 19.8%. Similar to the storage at room temperature, the degradation by any type of immobilized cells was similar.

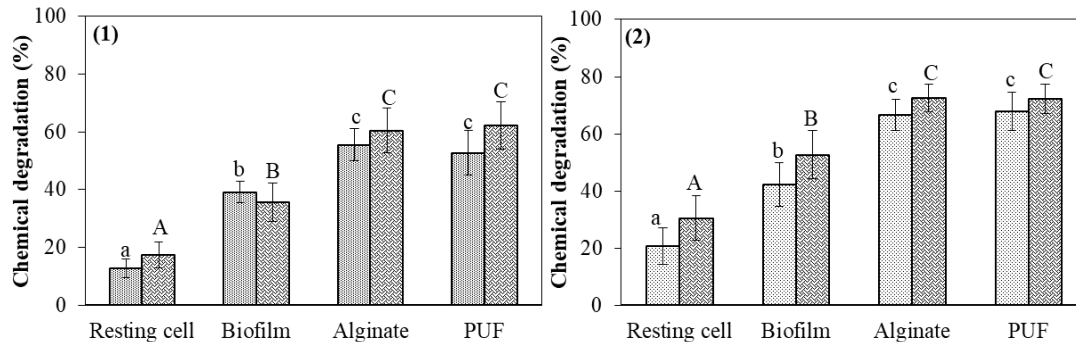


Figure 6. Degradation of chlorobenzene (▨) and 2-chlorotoluene (▩) by suspended and immobilized cells of the mixed-species of *C. testosterone* KT5 and *B. subtilis* DKT after 4-month storage at room temperature (1) and 4°C (2). Chlorobenzene and 2-chlorotoluene were added at 0.5 and 1.0 mM, respectively. Different small letters (a, b, c) indicate statistically significant differences in chlorobenzene degradation, while capital letters (A, B, C) indicate statistically significant differences in 2-chlorotoluene degradation ($p < 0.05$)

These results indicated that the immobilized cells showed better degradation in any condition and reduced adverse effects for long-term storage. Although a number of studies investigating the degradation of chlorobenzene and 2-chlorotoluene have been reported, no publication described the degradation by cells immobilized in PUF or alginate.

CONCLUSION

This study investigated the degradation of chlorobenzene and 2-chlorotoluene by mixed culture of *Comamonas testosterone* KT5 and *Bacillus subtilis* DKT. The degradation rates for these compounds of immobilized cells were higher than those of suspended cells. The immobilized cells also reduced adverse effects during storage. The results in this work showed a potential for application of immobilized cells as well as the mixed species for degradation of chlorobenzene and 2-chlorotoluene.

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