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Degradation of phenol and TCE using suspended and chitosan-bead immobilized *Pseudomonas putida*

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Abstract

The degradability of phenol and trichloroethene (TCE) by *Pseudomonas putida* BCRC 14349 in both suspended culture and immobilized culture systems are investigated. Chitosan beads at a size of about 1–2 mm were employed to encapsulate the *P. putida* cells, becoming an immobilized culture system. The phenol concentration was controlled at 100 mg/L, and that of TCE was studied from 0.2 to 20 mg/L. The pH, between 6.7 and 10, did not affect the degradation of either phenol or TCE in the suspended culture system. However, it was found to be an important factor in the immobilized culture system in which the only significant degradation was observed at pH >8. This may be linked to the surface properties of the chitosan beads and its influence on the activity of the bacteria. The transfer yield of TCE on a phenol basis was almost the same for the suspended and immobilized cultures (0.032 mg TCE/mg phenol), except that these yields occurred at different TCE concentrations. The transfer yield at a higher TCE concentration for the immobilized system suggested that the cells immobilized in carriers can be protected from harsh environmental conditions. For kinetic rate interpretation, the Monod equation was employed to describe the degradation rates of phenol, while the Haldane's equation was used for TCE degradation. Based on the kinetic parameters obtained from the two equations, the rate for the immobilized culture systems was only about 1/6 to that of the suspended culture system for phenol degradation, and was about 1/2 for TCE degradation. The slower kinetics observed for the immobilized culture systems was probably due to the slow diffusion of substrate molecules into the beads. However, compared with the suspended cultures, the immobilized cultures may tolerate a higher TCE concentration as much less inhibition was observed and the transfer yield occurred at a higher TCE concentration.

Keywords: Chitosan; Cometabolism; Immobilization; Phenol; Trichloroethylene (TCE)

1. Introduction

Trichloroethylene (TCE) has been widely used in industries as a solvent or a dry-cleaning agent, and is a common contaminant observed in soil and groundwater systems [1]. Because of its toxicity and potential carcinogenicity [2], TCE has been considered as a critical chemical of concern in soil and groundwater contamination sites.

TCE can be degraded biologically by reductive dechlorination under anaerobic conditions and by cometabolism under aerobic conditions [3]. Dalton and Stirling [4] defined cometabolism as "the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound" and suggested that non-growth substrates refer to "compounds that do not support cellular division." The first research to give attention to the cometabolic mechanism was by Leadbetter and Forster [5] and it concerned the oxidation of ethane by methane-utilizing bacteria. Wilson and Wilson [6] demonstrated that TCE can be converted to carbon dioxide by methanotrophic bacteria under aerobic conditions. In addition to methane, some aliphatics, such as ethane, propane, propene, butane, and isoprene [3], and aromatic compounds, such as toluene [7,8], creosol [3], and phenol [9], are effective substrates for cometabolizing TCE. In fact, Hopkins et al. [10] and Futamata et al. [11] have suggested that phenol may be the best substrate, due to its low toxicity and efficiency.

Immobilization or captured microorganism reactors in carriers may offer several advantages: (1) protection of microor-

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ganisms from harsh environmental conditions, (2) easier recovery from solution, (3) provision of higher cell density environment to increase overall substrate conversion rates, and, (4) reduced reactor volumes [12–14]. There are various technologies for bacteria immobilization, including bead entrapment, carrier binding, adsorption techniques, encapsulation, cell coating, and film attachment [12–15]. Uchiyama et al. [16,17] compared the cometabolic degradation of TCE using *Methylocystis sp.* immobilized in different matrix materials, including agarose, alginate, κ-carrageenan, polyurethane, photo-crosslinkable resin, and polyelectrolyte complex. The test results suggested that agarose is the best material for making the matrices due to its stability and higher degradability. Radway et al. [18] evaluated the potential of polyurethane embedded *Burkholderia cepacia* G4 for the degradation of TCE. The results demonstrated that polyurethane-embedded G4 cells can degrade TCE and benzene. Kneidel et al. [19] showed that the cells immobilized in the fibrous-bed bioreactor not only had a much higher TCE degradation rate, but also had a better tolerance to TCE toxicity.

Chitin, a polyaminosaccharide, is the second most abundant polymer in nature. It is the major component in the shell of crustaceans, the exoskeletons of insects, and the cell walls of fungi [20]. By the deacetylation process, chitin can be converted into another form of polyaminosaccharide, called chitosan. Chitin, the mother material of chitosan, is also a common group of solid waste in many countries. It has been reported that 50 to 90% of the total solid waste in the USA is from shellfish processing discards, and the total global annual estimate is around 5.12×10^6 metric tons [21]. Utilization of chitosan would provide the additional benefit of minimizing solid waste. There are three types of reactive functional groups in chitosan: an amino group as well as primary and secondary hydroxyl groups at the C-2, C-3, and C-6 positions, respectively [21]. This chemical structure makes chitosan easily modified as immobilization support compared to other materials. Both chitin and chitosan could serve as the material for making carriers for enzymes and cells [22,23]. Because of their nontoxicity and biocompatibility, the application of chitin and chitosan-based materials covers not only environmental engineering systems, but also food, pharmaceutical, medical, and agricultural industries.

Thus far, only a few attempts have been made to immobilize whole bacteria cells into chitosan beads. To our knowledge, there is no report on the degradation of TCE by chitosan-bead immobilized cells. The purpose of this study is to characterize the performance of the bacteria encapsulated in chitosan beads on the TCE degradation by the cometabolic mechanism. A purified strain of *Pseudomonas putida* was chosen in this study, and phenol was used for the carbon source. The strain was tested for the degradation of phenol and TCE under the cometabolic mechanism, both in suspended and immobilized forms. The performance of phenol and TCE degradation by *P. putida* was compared between the two forms. This may provide an insight into the feasibility of using chitosan beads to immobilize bacteria for the degradation of TCE.

2. Materials and methods

2.1. Chemicals

The growing medium used in this study contained KH_2PO_4 (420 mg/L), K_2HPO_4, (375 mg/L), $(NH_4)_2SO_4$ (244 mg/L), NaCl (30 mg/L), $CaCl_2$ (30 mg/L), $MgSO_4{\cdot}xH_2O$ (30 mg/L), and $FeCl_3{\cdot}6H_2O$ (3 mg/L). All the chemicals in the growing medium were analytical grade from Aldrich, US. Phenol (Riedel-deHaën, Germany) was added at 100 mg/L in the growing medium in all experiments as the sole carbon and energy source. The phosphate buffer solution (PBS) (Sigma, USA) contained 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl, and was used for diluting concentrated cells. The TCE used in this study was also analytical grade and purchased from J.T. Baker (USA). All the water used for dilution was from a Milli-Q water purification system (Millipore Corp., Bedford, MA, US). All media for experiment were autoclaved in advance.

2.2. Microorganism and growth

The bacteria chosen in this study was *Pseudomonas putida.* The purified strain, *P. putida* BCRC 14349, was purchased from Bioresource Collection and Research Center (BCRC), Taiwan. This bacterium grows on phenol, which is the sole carbon and energy source. The stock culture of the bacteria was stored in nutrient broth agar (Himedia, Agar powder) at 4 °C.

To recover the activity of the stock culture, one loop of bacteria from the culture-contained agar was transferred to 10 mL of the nutrient broth in a glass flask. The flask was then incubated in a water bath shaker (Wisdom, SB-9D, Taiwan) at 30 °C and 150 rpm for 6 h. After recovery, the bacteria were acclimatized. For the acclimatization, 5 mL of the bacteria-laden growing medium was transferred into a 250 mL serum bottle containing 100 mL of growing medium with 100 mg/L of phenol. The serum bottle was then maintained at 30 °C for 12 h in the water bath shaker at 150 rpm. To culture the acclimatized bacteria, 5 mL of the medium was then transferred into a 1000 mL serum bottle which contained 500 mL growing medium with 100 mg/L of phenol. Again, the serum bottles were incubated at 30 °C for 12 h in the water bath shaker at 150 rpm. Finally, the medium was centrifuged at $2000 \times g$ for 10 min, and the centrifuged pellets were harvested for further use.

2.3. TCE degradation in suspension systems

The centrifuged pellets of the phenol-cultured *P. putida* were used for the degradation of TCE in suspension-type experiments. In preparing the suspended cell cultures, centrifuged pellets were re-suspended with PBS, and then added into a 1,000-mL lateral modified serum bottle with 500 mL growing medium. To provide good control of the initial cell concentration for the experiments, the optical density at 550 nm (OD_{550nm}) in the solution was maintained at around 0.1 initially, in which the total cell mass in the solution is equivalent to 20 mg of cells

in dry weight. In addition, this OD_{550nm} at 0.1 is equivalent to 3×10^7 colony forming units per milliliter (CFU/mL). Two concentration ranges of TCE were used in the experiments: the low concentration range at 0.2–2 mg/L, and the high concentration range at 2–20 mg/L. It has been reported that the most common concentration of TCE in the field ranges from 0.05 to 5 mg/L [10]. In addition, because of the toxicity of TCE and its transformation byproducts, bio-degradation kinetics is inhibited as TCE concentration >10 mg/L [24]. Therefore, the TCE concentrations tested in this study covered the two concentration ranges. The TCE concentration in the solution was measured at a pre-determined time, and the gas phase TCE concentration was calculated according to the Henry's law [25]. The total TCE mass that remained in the system was calculated from the summation of TCE in both aqueous and gaseous phases. To determine the loss of TCE during the experimental period, blank experiments were also conducted under the condition of no carbon source provision. Changes of less than 5% of TCE mass were observed in the blank experiments, indicating that the loss during our experimental period was negligible. The initial pHs in the system were adjusted to predetermined levels at between 6.7 and 10 by adding either NaOH (1N, analysis grade, Merck, Germany) or HCl (1N, analytical grade, Merck, Germany).

2.4. TCE degradation in immobilized systems

To produce the immobilized chitosan beads, a chitosan stock solution was first prepared by dissolving 2.5 g chitosan powder (Koyo, degree of deacetylation = 77.8%, Japan) in 100 mL of water with 1 mL lactic acid (85%, J.T. Baker, Japan). Twenty mg of centrifuged pellets were re-suspended with 15 mL PBS, and then mixed with the chitosan solution in lactic acid to a final chitosan concentration of 1% (w/v). The *P. putida* cells and chitosan mixture was then added drop-wise through a 1 mL plastic tip into a 1% sodium tripolyphosphate (90–95%, Sigma, USA) solution for bead formation. To control the size of the beads, a constant height from the solution to the plastic tip was maintained. This allowed a constant size of 1–2 mm for the beads. After 30 min, the chitosan beads were separated from the solution and washed with 100 mL PBS for 10 min three times. The chitosan beads were then placed into the growing medium for further testing.

The beads were cross-linking with tripolyphosphate (TPP) under alkaline conditions, and would become unstable only if the pH was lower than 5. This pH value of 5 is lower than many ground water systems. The lifetime and reuse of chitosan are two important factors for practical application. A preliminary study showed that the beads could sustain degradation of phenol for several cycles and last for more than 1.5 months in column, suggesting the potential application of the beads.

Similar to the TCE degradation experiments in the suspended systems, two concentration ranges of TCE were also employed in the experiments for the immobilized system. The initial biomass, pH, and TCE concentration ranges used for both the suspension and the immobilized systems were all the same.

2.5. Analytical methods

To determine the concentration of biomass, optical density at 550 nm (OD_{550nm}) in the solution was measured with a spectrophotometer (Spectroquant NOVA 60, Merck). This wavelength falls between 420 and 650 nm which are commonly used to measure cell density [26]. In measuring the OD value, a 3 mL water sample was used. The OD values were compared with the spread-plate technique to obtain the colony forming units per milliliter (CFU/mL). Preliminary experiments suggested that a good correlation may be established between the OD value and colony forming units, reflecting that OD value may be used to represent the CFU on a volume basis. For the analysis of phenol and TCE, a 2 mL water sample was withdrawn from the lateral modified serum bottle and placed into a 5 mL glass vial with silicone/PTFE septum. 0.1 g L-(+)-Ascorbic acid (J.T. Baker, Taiwan) was then added to the sample to stop oxidation reactions, and 0.5 g NaCl (Riedel-de Haën, Germany) was added for salting for the solid phase micro-extraction concentration.

The concentration of phenol and TCE were determined using head-space solid phase micro-extraction (HSSPME) coupled with gas chromatography (GC). Water samples were first concentrated using HSSPME and then analyzed with a GC equipped with a flame ionization detector (FID) (HP 6890 series, USA). SPME is a simple and effective adsorption (or absorption) and desorption technique, which eliminates the need for solvents or a complicated apparatus, for concentrating volatile or non-volatile compounds in liquid sample or headspace [27]. A 75 μm Carboxen–PDMS phase fiber (Supelco, USA) was employed in the SPME system. The fiber was placed into the head space of a 5 mL sampling vial for the adsorption of phenol and TCE. During the extraction, the sampling vial was controlled at 40 °C and the adsorption time was 20 min. After the extraction, the fiber was injected into the GC inlet for analysis. The desorption time was set at 4 min and the temperature of the injection port was controlled at 220 °C. A DB-624 column (30 m × 0.320 mm, J&W Scientific, USA) was used for the separation. The oven temperature was held at 38 °C for 2 min, followed by an increase to 90 °C within 5.2 min and held for 6 min, and then increased to 180 °C within 4 min and held for another 3 min.

To view the inside of the bacteria-laden chitosan beads, microphotos were taken using a scanning electron microscope (SEM, S-3000N, Hitachi, Japan). Before analysis, the beads were first washed with a phosphate solution (15.25 g/L Na_2HPO_4, 5.85 g/L KH_2PO_4), fixed by 2.5% glutaraldehyde (25%, Merck, Germany), dehydrated by sequential ethanol (25%, 50%, 75%, 90%, 100%, Merck, Germany) extraction, and dried by a critical point dryer (HCP-2, Hitachi, Japan). The beads were then cut and coated with gold (ion sputter, E-1010, Hitachi, Japan) for analysis.

2.6. Kinetic analysis

Monod kinetics, as expressed as in Eq. (1), is commonly employed to simulate the kinetics of phenol biodegradation [3],

$$q_p = \left(\frac{dS}{dt}\right)\left(\frac{1}{X}\right) = -\left(\frac{kS}{Ks + S}\right) \quad (1)$$

where q_p is the specific substrate utilization rate (mg phenol $L^{-1}\,h^{-1}$ mg VSS^{-1}) for phenol, X is the biomass concentration (mg L^{-1}), k is the maximum specific rate of substrate utilization (mg phenol $L^{-1}\,h^{-1}$ mg VSS^{-1}), S is the substrate (phenol) concentration (mg L^{-1}), and Ks is the concentration giving one-half the maximum rate (or half saturation concentration) (mg L^{-1}).

The biomass concentration (X) at a specific time may be estimated from mass balance as expressed in Eq. (2),

$$X = X_0 + (S_0 - S)Y \tag{2}$$

where X_0 and S_0 represent the initial biomass and substrate concentrations, respectively, and Y is the yield (biomass produced per unit mass of substrate consumed).

Combining Eq. (2) and Eq. (1) gives the following:

$$\left(\frac{dS}{dt}\right) = -\frac{(kSX)}{(Ks+S)} = -\frac{[kS\,(X_0 + (S_0 - S)\,Y)]}{Ks+S} \tag{3}$$

which can be integrated over time for a batch reactor to yield the following relationship for time (t) and substrate concentration (S):

$$t = \left\{ KsY \ln\left(\frac{S_0}{S}\right) + (KsY + X_0 + S_0 Y) \ln\frac{[X_0 + (S_0 - S)Y]}{X_0} \right\} \{1/[kY(X_0 + S_0 Y)]\} \tag{4}$$

In Eq. (4), X_0, and S_0 are already known from the experiments, and Y may be either determined from independent experiments or from literature. Therefore, the two kinetic parameters, k and Ks, can be determined by comparing models (Eq. (4)) and observed values for S at different time t. Since only one initial phenol concentration (=100 mg/L) was tested in this study, this method accounting for all the data points obtained would allow for better kinetic determination. A spreadsheet method, based on weighted nonlinear least square analysis and commonly used for the evaluation of the integrated Monod equation, was employed [28]. In the analysis, Microsoft® Excel 2002 was used to solve the non linear problem.

The degradation of high concentration TCE is often described by Haldane's equation for accounting inhibition [11].

$$q_C = \left(\frac{dS_C}{dt}\right)\left(\frac{1}{X}\right) = -\frac{(k_C S_C)}{\left(Ks_C + S_C + S_C^2/K_i\right)} \tag{5}$$

where q_C is the specific substrate utilization rate (mg TCE $L^{-1}\,h^{-1}$ mg $VSS^{-1}\,h^{-1}$), k_C is the maximum specific rate of substrate utilization (mg TCE $L^{-1}\,h^{-1}$ mg VSS^{-1}) for TCE, S_C is the TCE concentration (mg L^{-1}), Ks_C is the concentration giving one-half the maximum rate (mg L^{-1}), and K_i is the inhibition constant (mg L^{-1}).

As the toxicity of TCE and its transformation byproducts may have influences on the degradation kinetics [24,41], several different TCE concentrations were tested in this study, and the initial-rates method was employed to solve k_C, Ks_C, and K_i in Eq. (5) [29]. Since K_i (the inhibition constant) is only important at higher TCE concentrations, the coefficients k_C and Ks_C were first solved simultaneously under low TCE concentrations. Once the best fitted k_C and Ks_C were determined, the K_i's were then solved under a higher TCE concentration condition. Commercially available mathematic software, POLYMATH [29], was employed to solve the non-linear regression kinetic model.

3. Results and discussion

3.1. The SEM photos of chitosan beads

Figs. 1 and 2 show the micro-photos of the surface and cross section of a *P. putida*-laden chitosan bead using SEM, respectively. As shown in the figures, the cells could grow well on the surface as well as in the internal parts of the chitosan bead. Based on the SEM microphotos, the size of the cells was about 1 μm × 0.5 μm, which is a typical size for bacteria. In addition, the cells were uniformly distributed in the whole bead, indicating that the bead may serve as a bio-reactor for the degradation of the target chemicals.

According to the SEM microphotos, the chitosan beads generally presented sub-micron pores. Since the beads are hydrogel-like, it was difficult to determine the porosity and the structure. Laboratory study showed that the beads may shrink after dehydration. Therefore, the beads need to be maintained in a water-saturated condition.

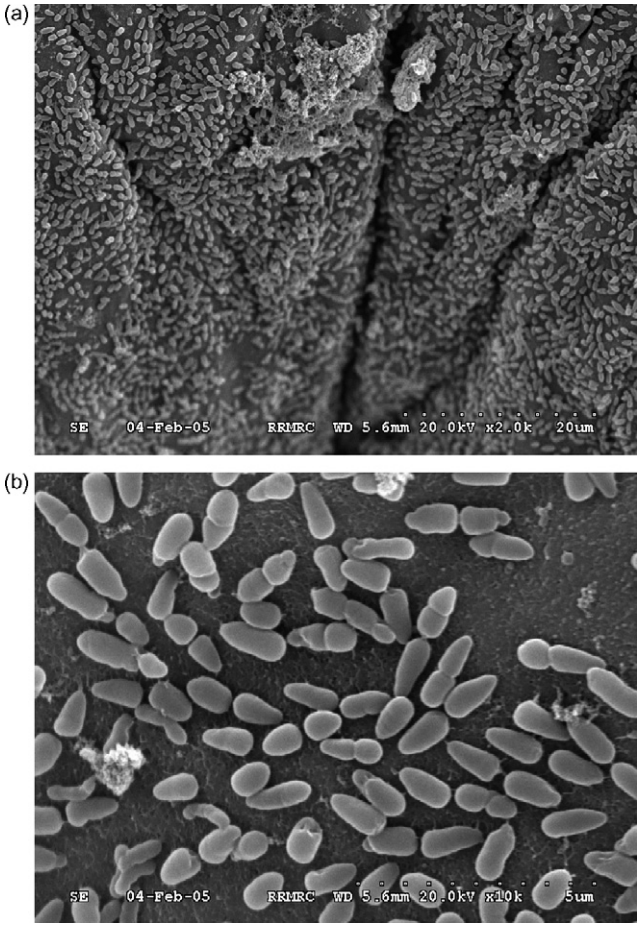

Fig. 1. The SEM micro-photos of the surface of a *P. putida*-laden chitosan bead, (a) magnified by 2000×, and (b) 10,000×.

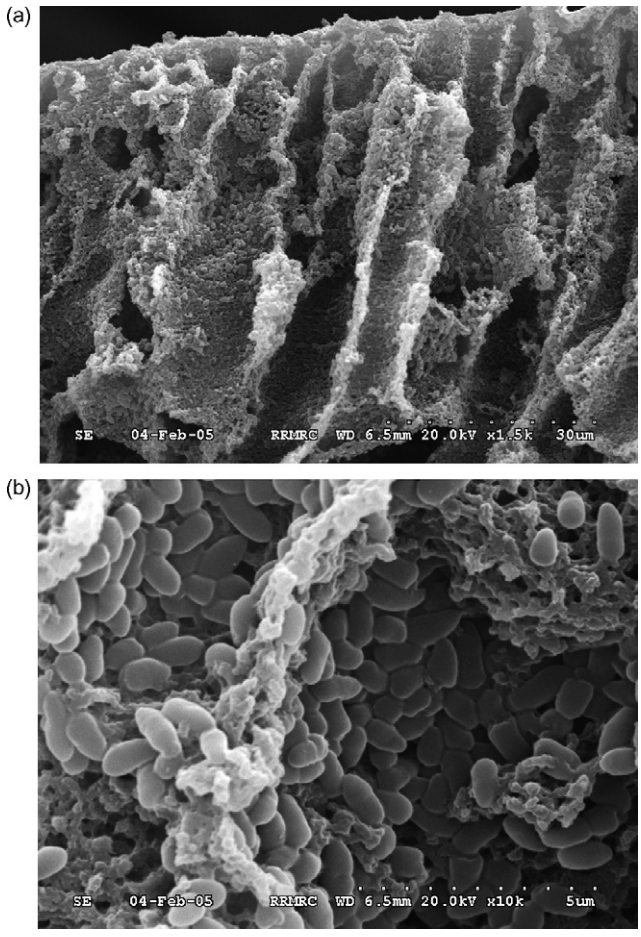

Fig. 2. The SEM micro-photos of the cross section of a *P. putida*-laden chitosan bead, (a) magnified by1500×, and (b) 10,000×.

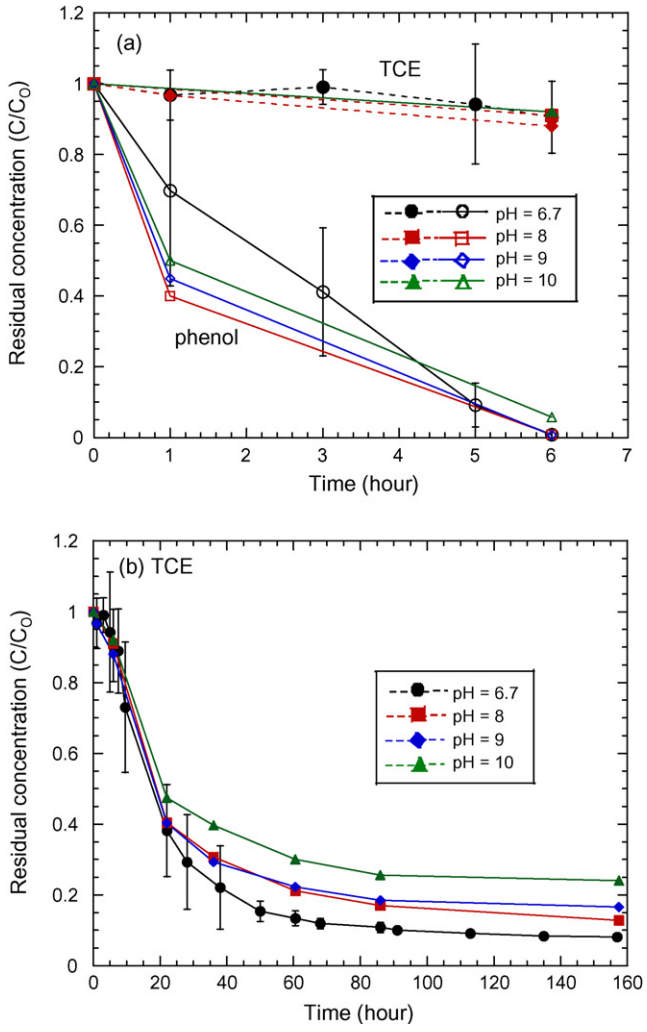

Fig. 3. Effect of pH on the degradation of phenol and TCE in a suspended cell culture, where (a) is phenol and TCE residual concentration for short time, and (b) TCE residual concentration for long time.

3.2. Effect of pH on phenol and TCE degradation

To obtain the optimum pH for the incubation of the *Pseudomonas putida* BCRC 14349 cells, both the suspended culture and immobilized culture were tested for their phenol and TCE degradation at pH values ranging from 6.7 (original pH for growing medium) to 10. In these experiments, the fed concentration of the carbon source, phenol, was fixed at 100 mg/L, while the initial TCE concentrations were controlled at 2 mg/L. The results are shown in Figs. 3 and 4 for suspended and immobilized cultures, respectively. For the suspended culture system, the pH value did not significantly influence the degradation of TCE and phenol. Little difference was observed for both phenol and TCE degradation among the four pHs tested. In addition, it was observed that the degradation of TCE began only after the exhaustion of phenol. Unlike that in the suspended culture system, pH is an important factor governing the degradation of the chemicals in the immobilized cell system. As illustrated in Fig. 4, phenol could be degraded well under a pH above 8. At pH = 6.7, the phenol concentration remained almost constant throughout the experiment, and, the degradation of phenol at pH = 7 only occurred after 62 h. In the experiments conducted at these two pH values, there was almost no TCE degradation. As shown in Fig. 4, only when pH was higher than 8, the immobilized cells started to degrade TCE, and the optimal pH for TCE degradation was 9.0. The low degradation of phenol at pH $\leq$7 may be attributed to the surface properties of chitosan beads. It has been suggested that the positively charged surface on chitosan may cause interactions with the negatively charged microbial cell membranes, leading to the leakage of intercellular constituents [30]. The acid dissociation constant (pK_a) of chitosan was reported to be about 6.2 to 7.0 [31]. When the pH is below pK_a, the amino group at the C-2 position of chitosan becomes positively charged. This creates a polycationic structure on the chitosan and may cause interaction with negatively charged cells [32]. In fact, titration of the chitosan bead surface tested in this study showed that the pH_{zpc} is around 7.0 to 7.5. Therefore, at pH levels lower than 7.5, the surface of chitosan may be positively charged. Thus, the activity of microorganisms would be suppressed at pH < ~7.5. Since pH is an important factor governing the degradation of phenol and TCE in the immobilized system, the degradation and kinetic tests in the later stages of this study were all conducted at pH = 9.

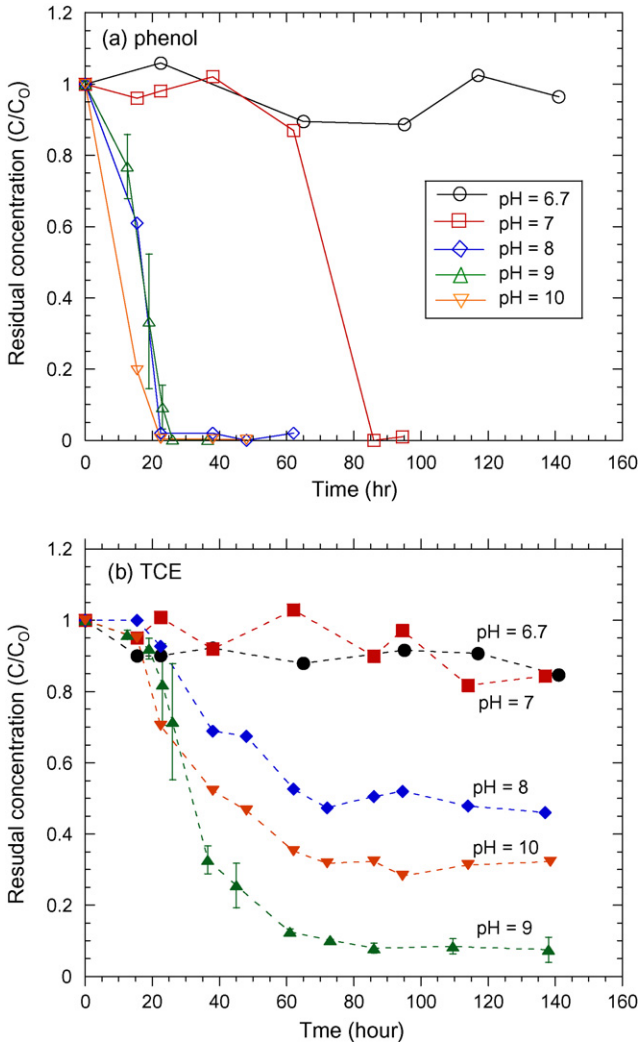

Fig. 4. Effect of pH on the degradation of phenol and TCE in an immobilized cell culture, where (a) is phenol residual concentration and (b) is TCE residual concentration.

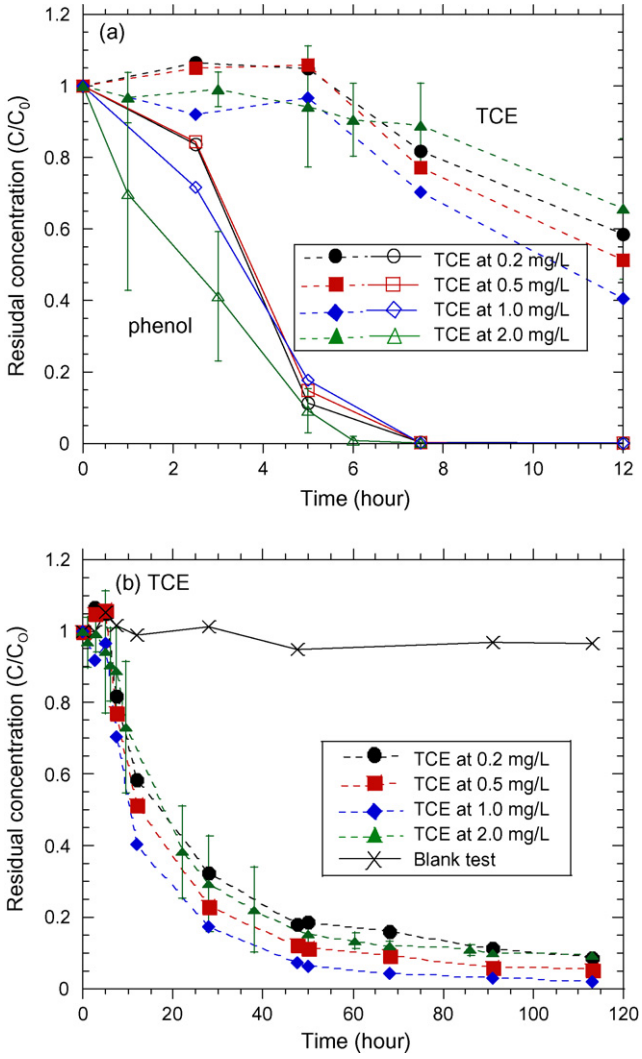

Fig. 5. The degradation of phenol (empty symbols) and TCE (solid symbols) at a low TCE concentration range for suspended culture systems, where (a) is phenol and TCE residual concentrations for a short time, and (b) is TCE residual concentrations for a long time.

3.3. Degradability of phenol and TCE in suspended systems

To evaluate the TCE degradability of *Pseudomonas putida* BCRC 14349, batch tests of suspended culture were conducted. The concentration of the carbon source, phenol, was fixed at 100 mg/L, while the TCE initially examined ranged from 0.2 to 2.0 mg/L. The batch test results are shown in Fig. 5. All the phenol in the four test runs were degraded within 5–7 h.

It may be worth pointing out that the degradation of TCE began only after the exhaustion of phenol, exhibiting a lag phase for the degradation of TCE. The same trends have also been observed by Futamata et al. [11]. In the *Comamonas testosterone* R5 culture, TCE degradation was initiated after phenol was completely degraded. One of the reasons for the lag time may be linked to the competition of the oxygenases responsible for the cometabolic effects in the system. This kind of competition between primary substrate and co-substrate has been reported [33–35]. For example, Kim and Hao [36] studied the cometabolic degradation of chlorophenols by *Acinetobacter* species. In their study, the degradation rate of the primary substrate, phenol, was affected by the relative concentration (to phenol) of 4-CP. This may suggest that there was a competition of oxgenases between phenol and 4-CP in their system. The lag time observed in this study may indicate that the affinity of phenol to the oxygenases responsible for the cometabolic effects in the system is overwhelmingly higher than that of TCE under the experimental conditions.

The studies relevant to cometabolic degradation of TCE were mostly in the concentration range of 0.05–5 mg/L [10]. The results mostly showed that the degradation rate decreased as TCE concentration increased, due to the adverse effect of toxicity. For example, Mu and Scow [24] have reported that as the initial TCE concentration was increased from 1 to 20 mg/L, the rate of toluene (a primary substrate) degradation decreased, and no TCE degradation occurred. To characterize the TCE susceptibility of the microorganism used in our study, a higher concentration range of TCE was employed in the degradation experiments. The phenol was kept at 100 mg/L, the same as that

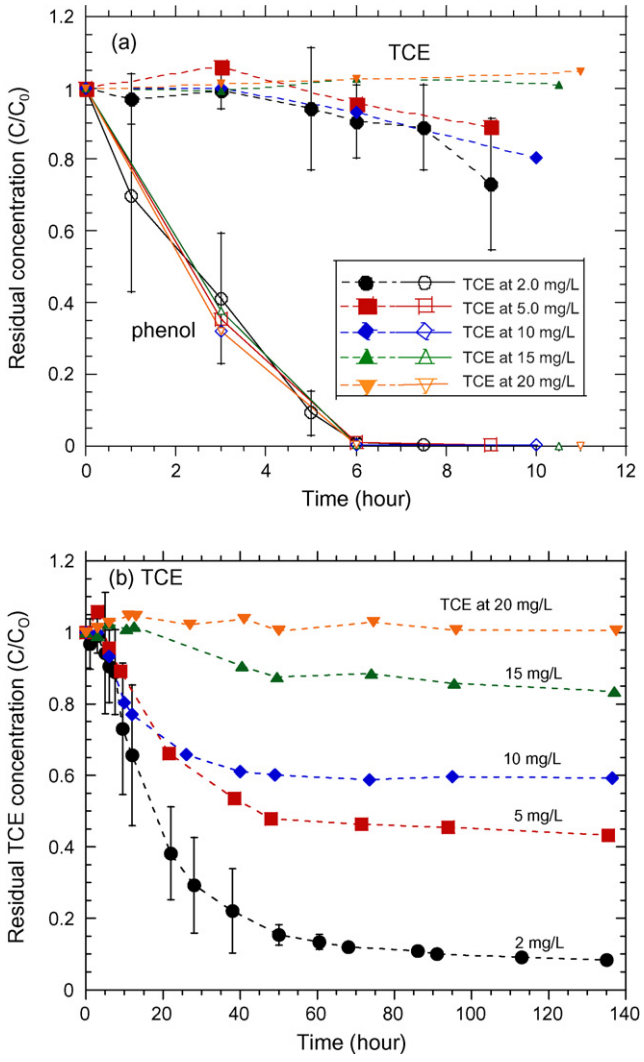

Fig. 6. The degradation of phenol (empty symbols) and TCE (solid symbols) at a high TCE concentration range for suspended culture systems, where (a) is phenol and TCE residual concentrations for a short time, and (b) is TCE residual concentrations for a long time.

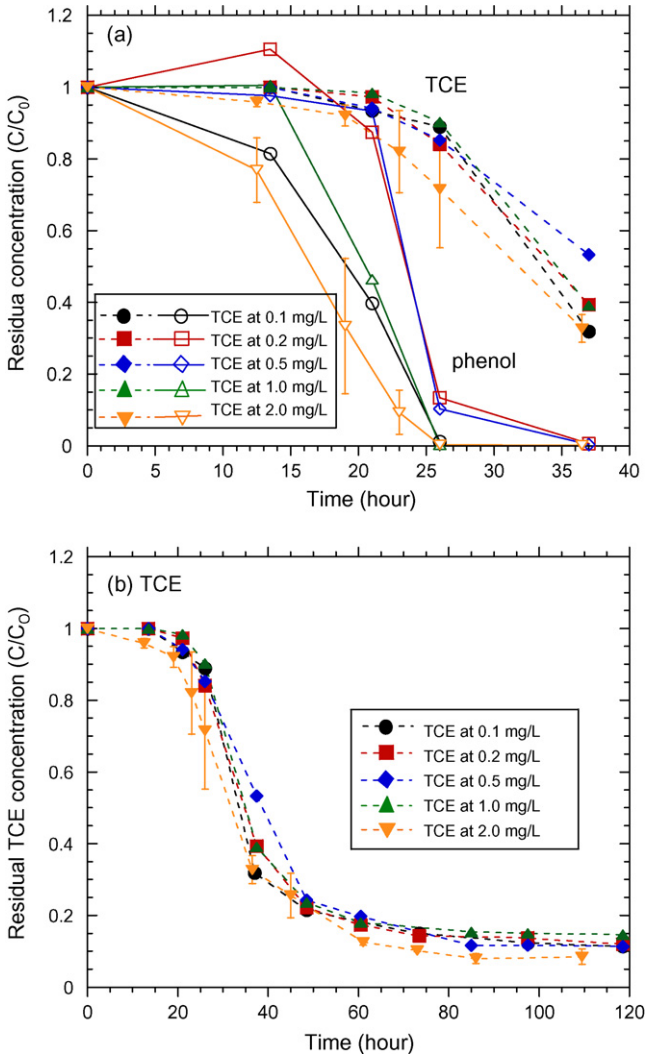

Fig. 7. The degradation of phenol (empty symbols) and TCE (solid symbols) at a low TCE concentration range for immobilized culture systems, where (a) is phenol and TCE residual concentrations for a short time, and (b) is TCE residual concentrations for a long time.

in the previous experiments, while TCE concentration increased by an order of magnitude (i.e., 2 mg/L to 20 mg/L). Under the high TCE concentrations, all the phenol in the five different tests was degraded within 6 h, which is similar to that in the low TCE concentration tests (Fig. 6). However, the degradation of TCE was strongly suppressed at higher TCE concentrations. This may be attributed to three probable reasons: limitation of oxygenases, toxicity caused by high TCE concentration, and deactivation of oxygenases, as suggested by other researchers [33–35].

3.4. Degradation of phenol and TCE in immobilized systems

The degradability of TCE by the cells immobilized in chitosan beads was also tested, and the results are shown in Figs. 7 and 8 for low and high TCE concentrations, respectively. Similar to the suspended culture cases, the degradation of TCE only started after the exhaustion of phenol, as shown in Fig. 7. However, compared with the suspended culture cases (Fig. 5), a lag time of about 12 h was observed before phenol degradation. This lag time may be due to the effect of mass transfer limitation. It has been reported that diffusion limitation plays an important role in immobilized bioreactors [37]. Although the chitosan beads are porous, some time may still be needed for phenol molecules to diffuse into the interior portion of the beads and to have the chance to contact the cells. In fact, the diffusion coefficient of phenol in the chitosan beads was estimated to be about 3.0×10^{-7} cm^2/s [38], which is about 30 times smaller than the diffusion coefficient of phenol in water ($\sim 9.0 \times 10^{-6}$ cm^2/s) [39]. The external (film) resistance was negligible in the system, as the incubator was shaken relatively rigorously and a Sherwood number of >200 was obtained for the system. Therefore, the degradation kinetics may be controlled by internal diffusion and/or reaction. To further analyze the importance of diffusion limitation in the system, Thiele's Modulus (ϕ)

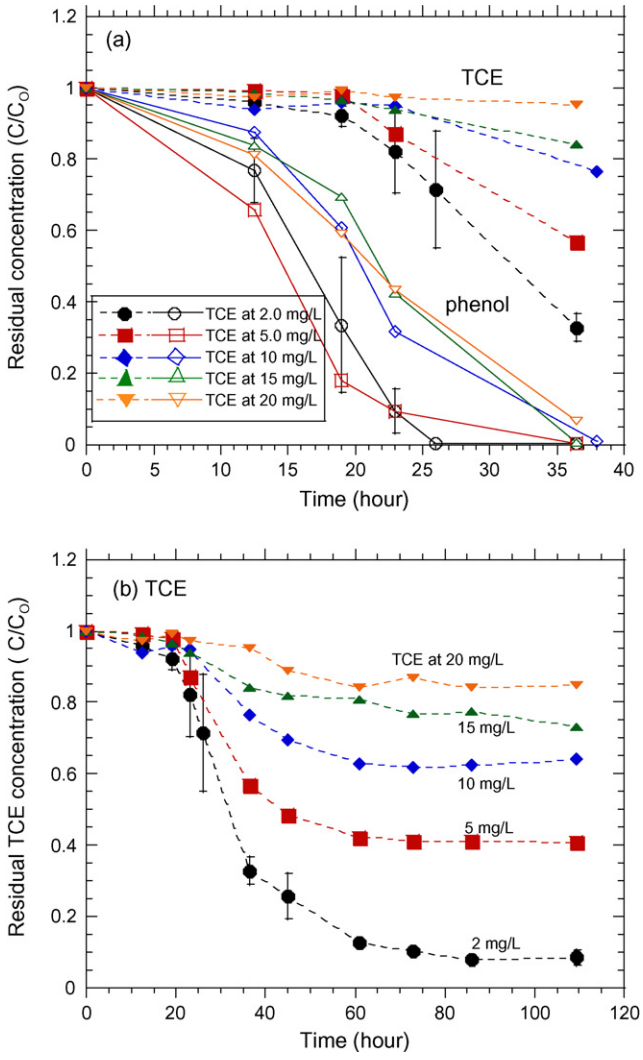

Fig. 8. The degradation of phenol (empty symbols) and TCE (solid symbols) at a high TCE concentration range for immobilized culture systems, where (a) is phenol and TCE residual concentrations for a short time, and (b) is TCE residual concentrations for a long time.

was calculated [37].

$$\phi = \frac{R_P}{3}\sqrt{\frac{\rho_P k_1}{D_e}} \quad (6)$$

where R_p = bead radius (0.1 cm), ρ_p = bacteria density in chitosan beads (=5.2×10^{-4} g cm^{-3}), and k_1 = first-order reaction rate constant for phenol (=5.0 cm^3 g^{-1} s^{-1}) [38].

For small ϕ, or $\phi < 0.5$, the system would be reaction controlled, and for $\phi > 5$, diffusion would be the limiting process [37,38]. In the system of chitosan beads and phenol, ϕ was around 3.1, indicating that both diffusion and reaction are important in the system.

Fig. 7 also shows that TCE degradation followed the same trend for all the five concentrations tested, suggesting that under the low TCE concentration range, the immobilized system performed similarly in degrading TCE. For the high initial TCE concentration cases, Fig. 8 shows a similar pattern of phenol degradation as that in Fig. 7. A lag time of 12 h was also observed

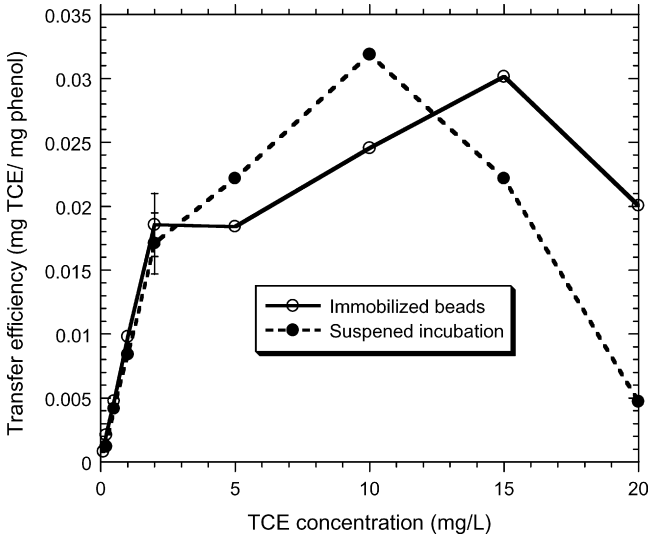

Fig. 9. The transformation efficiency of TCE for the suspended culture (solid symbols) and immobilized culture (empty symbols) systems.

in the figure. Unlike those for phenol, compared to Fig. 7 for low TCE concentration in immobilized systems, slower degradation rates of TCE at higher TCE concentrations were observed. This slower TCE degradation is similar to that in the suspended culture system under a high concentration of TCE, except that the encapsulated cells exhibit a longer (about 20 h) lag time for TCE.

To quantify the effectiveness of bacteria on the cometabolic degradation of TCE, a transfer yield proposed by Alvarze-Cohen and McCarty [40–41] is commonly used. They defined transfer yield (T_y) as the maximum mass of cometabolized compound that can be transformed by resting cells per unit mass of the primary substrate used for original cellular growth. In this study, a similar definition was used for characterizing the transfer efficiency at different TCE concentrations. Fig. 9 shows the TCE transfer efficiency at different TCE concentrations for both suspended and immobilized cultures. The transfer yield (T_y, maximum transfer efficiency) of TCE on a phenol basis was almost the same for the suspended and immobilized cultures (0.032 mg TCE/mg phenol), except that the yields occurred at different TCE concentrations. Semprini [42] has summarized transfer yield (T_y) for TCE transformation observed in laboratory and field studies. A wide range of T_y, 0.002–0.11 mg TCE/mg phenol, has been obtained. Our observation for T_y in both suspended and immobilized culture systems is in the range they reported. The T_y for the suspended culture was at 10 mg/L TCE concentration, and the transfer efficiency decreased sharply when the TCE concentration was larger than 10 mg/L, implying that TCE is toxic for the cometabolic enzyme in the suspended system. For the immobilized system, T_y occurred at a higher TCE concentration, and the transfer efficiency decreased only slightly with increasing TCE. This may suggest that the cells immobilized in the chitosan beads are protected from harsher environmental conditions, and have better tolerance to TCE toxicity. In addition, Fig. 9 also shows that the optimal phenol/TCE ratio is 10 (100 mg phenol/10 mg TCE) for the suspended sys-

tem, and 6.7 (100 mg phenol/15 mg TCE) for immobilized system. These values are in accordance with those reported by Shurtliff et al. [43], in which the optimal ratio was about 7.9 (210 mg phenol/26.7 mg TCE) for a mixed-culture chemostat system.

3.5. Comparison of phenol and TCE degradation kinetics in the systems

The two best fitted kinetic parameters for phenol degradation, the maximum specific rate of substrate utilization (k) and the half saturation concentration (Ks), are listed together with literature values in Table 1. Note that in the fitting of k and Ks, the yield (Y) was assumed to be equal to 0.42 g-VSS/g-BOD_L, as this value is typically used in aerobic systems [3]. As shown in Table 1, the best fitted k and Ks for suspended culture are 0.53 ± 0.22 mg phenol mg-VSS^{-1} h^{-1} and 5.71 ± 4.69 mg L^{-1} under the 95% confident interval, respectively. Molin and Nilsson [44] have tested the degradation of phenol using the same strain of bacteria as in this study. They observed that the k and Ks were 0.42 mg phenol mgVSS^{-1} h^{-1} and 3 mg L^{-1}, respectively, which is very similar to our k and Ks. For the immobilized culture tested in this study, the best fitted parameters are 0.082 ± 0.04 mg phenol mgVSS^{-1} h^{-1} and 5.05 ± 8.16 mg L^{-1} for k and Ks under the 95% confident interval, respectively. Table 1 also lists the values of k/Ks, which is a representation of a first-order kinetic rate constant under low substrate concentration for Eq. (1). The k/Ks for immobilized culture systems are only about 1/6 of those for the suspended systems, suggesting slower kinetics for the immobilized culture systems.

Neglecting the lag phase, the degradation rate of TCE can be simulated by Haldane's equation as shown in Eq. (5). The best fitted coefficients for k_C and Ks_C are 0.017 ± 0.0066 mg TCE mgVSS^{-1} h^{-1} and 9.90 ± 4.34 mg L^{-1}, respectively, for the suspended culture, and are 0.00036 ± 0.00026 mg TCE mgVSS^{-1} h^{-1} and 0.51 ± 0.62 mg L^{-1}, respectively, for the immobilized culture, all under the 95% confident intervals. The inhibition constant, K_i, was estimated to be 2.1 mg/L for the suspended cultures, while no constant K_i values could be obtained for the immobilized culture system. Similar to the reported values shown in Table 1, K_i in this study is always >400 for the immobilized cultures. As suggested by other researchers [11,45], larger K_i is a reflection of no inhibition effect for TCE degradation. This may indicate that the immobilized cultures tolerate higher TCE concentrations than suspended culture systems. The k_C to Ks_C ratio, a representation of a first-order degradation rate constant, for immobilized systems was about 1/2 of that for the suspended systems. Although the degradation of TCE in the immobilized systems is slower, the Ks_C was also smaller compared to that in suspended cultures, as shown in Table 1. A lower Ks_C may indicate a stronger affinity of oxygenases for TCE [26,45]. Therefore, the reason for lower kinetic rates at lower concentrations (k_C/Ks_C) for the immobilized systems is the smaller maximum specific rate of substrate utilization (k_C).

In the degradation of phenol and TCE in the immobilized culture systems, phenol exhibited much slower rates ($k/Ks \sim 1/6$ for

Table 1
The estimated rate constants for phenol and TCE degradation

Microorganism	Phenol			TCE				Ks/Ks_C	References
	k (mg phenol mg VSS^{-1} h^{-1})	Ks (mg L^{-1})	k/Ks (L mgVSS^{-1} h^{-1})	k_C (mg TCE mgVSS^{-1} h^{-1})	Ks_C (mg L^{-1})	k_C/Ks_C (L mgVSS^{-1} h^{-1})	K_i (mg L^{-1})		
BCRC 14349 Suspended culture	0.53 ± 0.22	5.71 ± 4.69	0.093	0.017 ± 0.0066	9.90 ± 4.34	0.0017	2.13 ± 1.59	0.58	This study
Chitosan Immobilized culture	0.082 ± 0.04	5.05 ± 8.16	0.016	0.00036 ± 0.00026	0.51 ± 0.62	0.0007	>400	9.9	This study
P. putida P-6	0.09 ± 0.016	0.37 ± 0.16	0.24	0.044 ± 0.014	103.5 ± 57.64	0.00043	>210	0.0036	11
P. putida BH	0.113 ± 0.0085	0.3 ± 0.04	0.38	0.0024 ± 0.0006	34.06 ± 7.86	0.00007	>32.75	0.0088	11
Comamonas testosterone R5	1.52 ± 0.068	0.0376 ± 0.0094	40.42	0.0079 ± 0.0008	1.44 ± 0.24	0.0055	>72.05	0.026	11
Variovorax sp. Strain c24	0.52 ± 0.037	0.77 ± 0.11	0.67	0.0094 ± 0.00078	0.45 ± 0.14	0.021	2540 ± 1270	1.71	45

immobilized systems of that for suspended systems) compared to TCE ($k_C/Ks_C \sim 1/2$). The lower kinetic rate of substrate utilization in immobilized systems may be due to the slow diffusion of substrate molecules into the beads, particularly for phenol. For TCE, there was a lag time before degradation. Therefore, TCE molecules may have enough time to diffuse into the beads. As phenol was degraded and oxygenases were produced, the TCE molecules were already present in the adjacent area. Under this condition, compared with phenol molecules, TCE molecules required much less time to contact the bacteria and oxygenases.

The kinetic coefficients range diversely as shown in Table 1. Futamata et al. [11,45] have isolated and characterized some phenol-degrading and TCE-degrading bacteria. A relationship between the *Ks* of phenol and TCE is summarized, and divided into four groups. Three of the four groups include: the low-*Ks* group, such as *Comamonas testosterone* R5, the moderate-*Ks* group, such as *Pseudomonas putida* BH, and the high-*Ks* group, such as *P. putida* P-6 (Table 1). A positive correlation between *Ks* for phenol and Ks_C for TCE was found for the three groups, where bacteria exhibiting high *Ks* values for phenol showed high Ks_C values for TCE. A fourth group, the *Variovorax* group, was characterized as high *Ks* values for phenol but low Ks_C values for TCE. Unlike the four groups of bacteria, the bacteria strain employed in this study shows high *Ks* values for phenol and moderate Ks_C values for TCE. The Ks/Ks_C values, which mean the oxygenase competitive ability between TCE and phenol, are also summarized in Table 1. Larger Ks/Ks_C values represent a stronger affinity of oxygenase for TCE, and would allow the competitive inhibition of TCE degradation in cometabolic systems. The bacteria strain employed in this study also possesses a larger Ks/Ks_C compared to the first three groups of bacteria.

4. Conclusions

A microorganism, *Pseudomonas putida* BCRC 14349, was employed to degrade both phenol and TCE under suspended and chitosan bead immobilized systems. Based on the scanning electron microscope (SEM) microphotos, the *P. putida* cells grew well on both the surface and interior of the immobilized media, and the cells were uniformly distributed in the whole bead. The degradation experiments showed that both the primary substrate, phenol, and cometabolic secondary substrate, TCE, were able to degrade at the tested concentrations, phenol = 100 mg/L, and TCE = 0.2–20 mg/L. The effect of pH, between 6.7 and 10, on the degradation of both phenol and TCE may be neglected for the suspended culture system. However, for the immobilized culture system, phenol and TCE degradation were only observed at pH >8. The different effect of pH on the degradation may be linked to the surface properties of the chitosan beads and its interaction on the activity of the bacteria. In the degradation experiment, the degradation of TCE began only after the exhaustion of phenol, indicating that the competitiveness of phenol is large than TCE. The maximum transfer yield of TCE was almost the same for the suspended and immobilized cultures (0.032 mg TCE/ mg phenol). However, the maximum transfer yields for suspended and immobilized systems occurred at different TCE concentrations. The transfer yield at higher TCE concentrations for the immobilized system may suggest that the cells immobilized in carriers were provided protection from harsh environmental conditions, and had a better tolerance to the toxicity of TCE. The first-order kinetic rate constants (k/Ks) for phenol degradation in immobilized culture systems were only about 1/6 of those in the suspended systems, while those (k_C/Ks_C) for TCE were 1/2. The slower kinetics for the immobilized culture systems may be due to the slow diffusion of substrate molecules into the beads. Although having slower kinetics, a much large inhibition constant (K_i) was observed for the immobilized culture systems, suggesting that the immobilized cultures may sustain higher TCE concentrations compared with suspended cultures.

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