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Performance of Oxygen-Carbon-Inducer Releasing Material for Biodegradation of Trichloroethylene, *cis*-Dichloroethylene and Vinyl chloride

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Abstract: Performance of oxygen-carbon-inducer releasing materials (OCIRM) on co-metabolize TCE and degradation of its metabolites were studied. Potential of the orange peel as the inducer was evaluated as compared to limonene solution. The experiment was conducted in batch mode to determine the optimum size of OCIRM and suitable carbon source. Results showed a 1.0 cm^3 cube was the optimal size and sugarcane bagasse was a suitable C source in which the maximum organic releasing rate of 0.30 mg COD day^{-1} g^{-1} cube and dissolved oxygen concentration of 11.95 mg O_2 L^{-1} were generated. Limonene in orange peel and in solution successfully induced monooxygenase enzyme to co-metabolize TCE. Optimal concentration ratio of contaminant to limonene was 1:2.5. OCIRM containing sugarcane bagasse and limonene in orange peel using a biofilm of aerobic sludge as the inoculum effectively co-metabolize TCE, *cis*-DCE and VC with maximum percentage removal of 56, 61 and 72%, respectively.

Key words: Co-metabolism, limonene, *Rhodococcus gordoniae* P3

INTRODUCTION

Chlorinated aliphatic hydrocarbons, especially trichloroethylene (TCE), are the most widespread contaminants in groundwater. TCE has been a concern due to its toxicity, especially carcinogenic effect and high persistence in the environment. Under anaerobic condition, such as in groundwater, TCE could be degraded via reductive dechlorination process resulting in the metabolites named *cis*-1,2-dichloroethylene (*cis*-DCE) and Vinyl Chloride (VC) which have the higher toxicity than TCE. Due to the slow degradation rate under anaerobic condition, *cis*-DCE and VC have been accumulated and detected in many TCE contaminated sites (Broholm *et al*., 2005; Shen and Sewell, 2005). Therefore, the interests have been paid to the aerobic bioremediation which is effective in degradation of TCE and its metabolites especially *cis*-DCE (Olaniran *et al*., 2008).

Under aerobic condition, the biodegradation of TCE and *cis*-DCE could occur via co-metabolism process in which the primary substrate mainly toluene and phenol are used as their growth substrates and act as the inducer of monooxygenase enzyme capable of co-metabolic oxidation of TCE and its metabolites (Ferhan, 2003; Kim *et al*., 2008). However, toluene and phenol are toxic to the environment. Therefore, there are the attempts to search for the plant terpenes such as cumene, limonene, carvone and pinene which have analogous structures to toluene and phenol for inducing microbial degradation of TCE (Singer *et al*., 2003; Suttinun *et al*., 2004, 2009). In this study, orange peel was used to induce the production of monooxygenase enzyme in microorganisms since it composes of limonene (>90%) that might effectively induce the cometabolism degradation of TCE (Raeissi and Peters, 2004).

In order to sufficiently supply the primary substrate and oxygen throughout the *in situ* remediation process of TCE in groundwater, researchers have developed the organic-oxygen- releasing materials containing different sources of oxygen and/or primary substrates to be used as the passive oxygen and primary substrate supplier

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during TCE remediation in groundwater (Borden *et al*., 1997). The results clearly demonstrated that with the presence of these slow release materials, the aerobic co-metabolism of TCE and its metabolites could be efficient for long term of remediation process and the contaminant plume in groundwater could be effectively confined (Borden *et al*., 1997; Kao *et al*., 2001).

The objective of this study is to construct the oxygen-carbon-inducer-releasing material (OCIRM) and evaluated its performance for co-metabolism of TCE and degradation of its metabolites. The OCIRM contained CaO_2, sugarcane bagasse or compost, limonene as the oxygen, carbon and inducer sources, respectively. It is hoping that the OCIRM might be able to continuously release oxygen, carbon and limonene when contact with groundwater to stimulate and induce the microorganisms to completely degrade TCE contamination in groundwater.

MATERIALS AND METHODS

Research location: Construction of the OCIRM and evaluation of its performance for co-metabolism of TCE were carried out at Department of Biotechnology, Faculty of Technology, Khon Kaen University, Thailand. Study on the performance of OCIRM on degradation of *cis*-DCE and VC was carried out at Department of Environmental Engineering and Science, Chia Nan University of Pharmacy and Science, Tainan, 71710, Taiwan, Republic of China. This study was conducted between June, 2008-April, 2009.

Chemicals: TCE, 98%, was purchased from Riedel-deHa¸n, Germany. *cis*-DCE, 97%, was purchased from Sigma Aldrich Chemical, USA VC, 2000 mg L^{-1} in methanol, was purchased from Chem Service, USA R-(+)-limonene, 98%, was purchased from Fluka, Switzerland. All other chemicals and reagents are analytical grade and purchased from BDH, England.

Synthetic groundwater: Synthetic groundwater consisted of (mg L^{-1}): KH_2PO_4, 326.4; Na_2HPO_4, 1263.8; $Mg_2SO_4.7H_2O$, 98.6; $CaCl_2.2H_2O$, 44.1; NH_4Cl, 10.7 and trace elements including $FeSO_4.7H_2O$, 1; $MnSO_4.4H_2O$, 1; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.25; $Na_2B_4O_7.10H_2O$, 0.25; $CoCl_2.6H_2O$, 0.25; $CuCl_2.2H_2O$, 0.25; $ZnCl_2$, 0.25; NH_4VO_3, 0.1. The pH of the synthetic groundwater was 7.5 (Kao *et al*., 2001).

Potential inducer: Orange peel, the waste from orange juice drink shop, was used as potential inducer for co-metabolism of TCE and *cis*-DCE by *Rhodococcus gordoniae* P3 and aerobic sludge in comparison to limonene solution. It was dried at room temperature, chopped into small pieces, passed through 2 mm sieve and kept at -20°C prior the usage. One gram dry weight of orange peel contains approximately 13 mg of limonene determined by the method followed by Chotratanadilok *et al*. (2002).

Organic amendment: Compost and sugarcane bagasse were used as carbon source in this experiment. Compost was obtained from Faculty of Agriculture, Khon Kaen University. It was composted from yard waste. The compost sample was pulverized and passed through a 2 mm sieve. It consists of 0.76% total N and 7.44% organic carbon.

Sugarcane bagasse, the fibrous residue of sugarcane stalk which remain after the juice was extracted, was collected from sugar factory, Mitara Phoo Weang Co Ltd., Khon Kaen Province. Before the usage, sugarcane bagasse was dried at 60°C, milled and sieved through a 2 mm sieve. The dry milled sugarcane bagasse consists of 0.19% total N and 49.93% organic carbon.

Support material: Corncob was used as the support material to immobilize the microorganisms. It was obtained from Faculty of Agriculture, Khon Kaen University, Thailand. Organic carbon and total nitrogen of corncob were 45.84 and 0.23%, respectively. It was shredded by knife into small pieces (approximately 0.5×0.5×0.5 cm). After that, it was delignified by boiling in 1% NaOH for 3 h to remove lignin which might be toxic to microorganisms and then thoroughly washed under tap water and soaked in distilled water overnight (Iconomou *et al*., 1995). This process was done 2 times and kept at -20°C prior the usage.

Inoculum

Pure culture: TCE degrader, *R. gordoniae* P3 (GenBank accession number EF450777), a gram positive aerobic bacteria, was isolated from petroleum-contaminated soil collected from Bangkok area. It was kindly provided by Dr. Ekawan Luepromchai, Department of Microbiology, Chulalongkorn University, Thailand.

Aerobic sludge: Aerobic sludge was collected from wastewater treatment plant of Lardkabang Industrial Sector, Bangkok, Thailand. Wastewater treated at this wastewater treatment plant is from electronic part industries where TCE and toluene are used as solvent. Aerobic sludge was acclimatized with 10 mg L^{-1} TCE and 10 mg L^{-1} limonene solution, shaken at 150 rpm and incubated at the room temperature for 21 days (Ozbelge *et al*., 2007) to enhance TCE degradation ability

of the microorganisms in sludge. After acclimatization, the aerobic sludge was harvested by centrifugation at 6,000 rpm for 10 min and the pellets were re-suspended in synthetic groundwater before using as the seed inoculum.

Immobilization of *R. gordoniae* P3 on corncob: Cell immobilization by adsorption mechanism was conducted by adding 75 g of delignified corncob into 300 mL Minimal Salt Medium (MSM) (Focht, 1994) and autoclaved at 121°C for 15 min twice before inoculating with 10% (V/V) of *R. gordoniae* P3 (10^6 cfu mL^{-1}) or aerobic sludge (20,600 mg VSS L^{-1}). Then, 172 mg L^{-1} of toluene, a primary substrate, was added into the bottle before incubating at room temperature, shaken at 200 rpm on orbital shaker for 24 h. After that, the immobilized cells were transferred to a fresh MSM containing 172 mg L^{-1} toluene and incubated as described previously for 2 times before harvesting by filtration through Buchner filter funnel. The immobilized cells on corncob were washed twice with 0.85% NaCl using aseptic technique. The immobilized cells were furthered cultivated in MSM containing 10 mg L^{-1} limonene for 7 days, shaken at 200 rpm on orbital shaker, at room temperature. Immobilized cells were harvested and washed as previously described then kept at 4°C until the usage. The internal cell density on the corncob was approximately 10^6 cells g^{-1} dry wt of support materials determined by viable plate count technique.

Biofilm: Biofilm of *R. gordoniae* P3 and aerobic sludge was developed by coating the inoculum on a 1.0 cm^3 cube oxygen-carbon-inducer-releasing material (OCIRM) using the method adapted from the protocol of Tremoulet *et al.* (2002). Briefly, five gram of 1.0 cm^3 cube OCRM was placed in a 50 mL serum bottle containing 20 mL of synthetic groundwater. The bottles were inoculated with 10% inoculum (*R. gordoniae* P3 (10^6 cfu mL^{-1}) or aerobic sludge (20,600 mg VSS L^{-1}) and further incubated for 3 weeks at room temperature. After 3 weeks, the synthetic groundwater was removed and biofilm was used in the experiment. Extraction and quantification of bacteria in biofilm was conducted following the method of Kadurugamuwa *et al.* (2003).

Oxygen-carbon-releasing material (OCRM): The purpose of OCRM was to continuously supply the dissolved oxygen (DO) and carbon for microorganisms to use in co-metabolism of the contaminants. It was comprised of binding cement, calcium peroxide (CaO_2), sand, carbon source (compost or sugarcane bagasse), fly ash and water at the ratio of 1.5:1.5:0.2:0.2:1.3:2 by weight (modified from Kao *et al.*, 2001). When the inducer (limonene in orange peel or limonene solution) was added into the OCRM, it was called the Oxygen-Carbon-Inducer-Releasing Material (OCIRM).

Effect of OCRM size on supplying dissolved oxygen and carbon: Various sizes of OCRM of 1x1x1, 2x2x2 and 3x3x3 cm^3 cube were evaluated in order to test the effect of OCRM size on supplying dissolved oxygen and carbon over time. Sugarcane bagasse and compost were assessed its effectiveness as carbon source. Inducer was not added. The experiment was conducted in a 1 L enclosed glass bottle containing 125 g of cube and 250 mL of DI water. Cube without CaO_2 and carbon source was used as a control. Water samples were taken every day for 100 days and analyzed for the Dissolved Oxygen (DO) and COD. DO was measured by DO meter (DO 551, Digital, Japan). COD analysis was conducted by dichromate reflux method (APHA, 1992; Nkegbe *et al.*, 2005).

Effect of concentration ratio of TCE to limonene on co-metabolism efficiency of TCE by *R. gordoniae* P3 and aerobic sludge: The effect of concentration ratio of TCE to limonene on the co-metabolism efficiency of TCE by *R. gordoniae* P3 and aerobic sludge was investigated. In this experiment, limonene in orange peel and limonene solution were added into OCIRM as the inducers at various concentrations ratio of TCE to limonene of 1:0.5, 1:1, 1:2.5 and 1:5. A suitable carbon source was added into the OCIRM. Experiment was conducted in a 50 mL serum bottle containing 20 mL synthetic groundwater, 10 mg L^{-1} TCE, 5 g OCIRM and 10^6 cfu mL^{-1} of inoculum (*R. gordoniae* P3 or aerobic sludge). The bottle was tightly sealed with Teflon-lined rubber septa and aluminum cap to prevent the leakage of TCE into the air. Controls were non-inoculated and inoculated microcosms without OCIRM. Water samples were taken at day 0, 5, 7, 14, 21 and 35 to analyze for the TCE residues by GC-FID.

Effect of inoculum form on co-metabolism of TCE: Efficiency of various forms of aerobic sludge and *R. gordoniae* P3 i.e., free cell, immobilized cell on corn cob and biofilm coated on OCIRM to co-metabolism TCE were conducted. Suitable concentration ratio of TCE to limonene and suitable carbon source were added into the OCIRM. Microcosms were constructed in a similar manner as previous experiment except the inoculum was added in various forms.

Biodegradation of *cis*-DCE, VC and a combination of *cis*-DCE and VC: Co-metabolism of *cis*-DCE, VC and a combination of *cis*-DCE and VC by the most effective type

and form of inoculum were conducted. Suitable concentration ratio of TCE to limonene and suitable carbon source were added into the OCIRM. Microcosms were constructed in a similar manner as previous experiment except the synthetic groundwater contained 10 mg L^{-1} *cis*-DCE or 10 mg L^{-1} VC or a combination of 10 mg L^{-1} *cis*-DCE and 10 mg L^{-1} VC. The samples were sacrificed at day 0, 5, 7, 14 and 21 and analyzed for *cis*-DCE and VC by GC-FID and chloride ion by IC.

Enumeration of toluene degrading bacteria: One gram wet weight of immobilized support material was blended by blender into small particles under aseptic condition. Then, serial 10-fold dilutions of each suspension were plated on Minimal Salt Agar (MSA) that coated with 10 mg L^{-1} of the contaminants and incubated at room temperature in the box fumigated with toluene as a primary substrate for one week. The number of colony forming unit (cfu) between 30-300 colonies in each plate were counted.

Analytical method: TCE, *cis*-DCE and VC concentrations in the synthetic groundwater were measured by analyzing 50 μL headspace sample on Agilent 4820D gas chromatography equipped with flame ionization detector (GC-FID) and Rtx-624 capillary column (30×0.53 mm ID; 3 μm). The GC oven temperature of 60°C was held for 6 min and then ramped at 8°C min^{-1} to 200°C where the oven was held for 1 min. Percentage of recovery for this analyzing method is 87%. The detection limit of GC-FID is 0.1 mg L^{-1} for TCE, *cis*-DCE and VC.

Anions i.e., bromide and chloride were determined by Ion Chromatograph (Dionex DX-120) equipped with RFC-30 EGCII (KOH), Autosampler Thermo Finnigan Spectra SYSTEM model AS 1000 with 20 μL injection volume, IonPac®AG11 guard column (4×50 mm), IonPac®AS11 analytical column (4×250 mm), ASRS®-ULTRA II (4 mm) suppressor and conductivity detector. The column temperature was controlled at 30°C. IC was operated at the flow rate of 1.0 mL min^{-1} with gradient of 0.5 mM KOH 0-4 min, 0.5-35 mM KOH 4-22 min, 18 mM KOH 22-26 min and 0.5 mM KOH 26-30 min.

RESULTS AND DISCUSSIONS

Performance of OCRM: The carbon and oxygen released from the OCRM into the DI water were determined as COD and DO, respectively. The results showed that the overall COD releasing rate of each carbon source at all sizes dropped rapidly within 12 days and then stable after day 15 (Fig. 1). Every size of the OCRM containing sugarcane bagasse gave COD releasing rates higher than that of

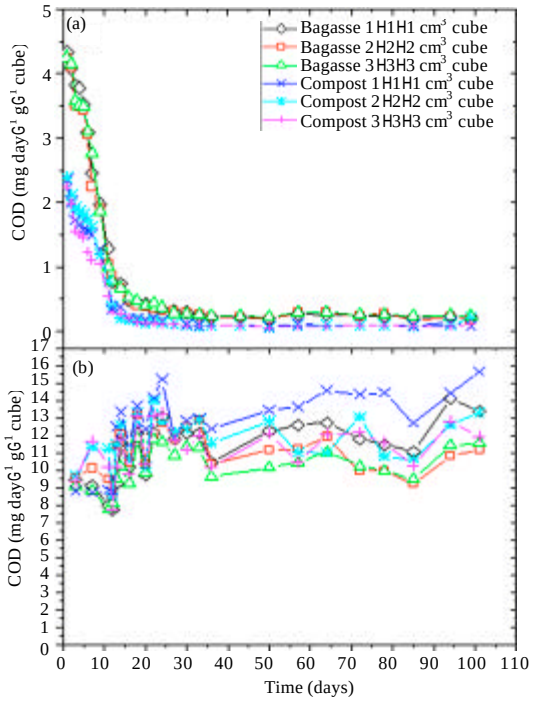

Fig. 1: (a) COD releasing rate from oxygen releasing material and (b) DO concentration from oxygen releasing material

compost (Fig. 1a). However, there was no significantly different on COD releasing rates obtained among the size of the OCRM containing sugarcane bagasse or compost. The maximum COD releasing rates of 0.30 and 0.12 mg COD day^{-1} g^{-1} cube were obtained from a 1.0 cm^3 of OCRM containing sugarcane bagasse and compost, respectively.

Size and carbon source in the OCRM did not affect DO concentration (Fig. 1b). The average maximum oxygen concentrations over 100 days were 13.55 mg O_2 L^{-1} and 11.95 mg O_2 L^{-1} obtained from a 1.0 cm^3 cube OCRM containing compost and sugarcane bagasse as C source, respectively.

Since the results indicated that a 1.0 cm^3 cube with sugarcane bagasse as a C source showed a maximum COD releasing rates and DO concentration, therefore a 1.0 cm^3 was used in a further experiment to supply oxygen and carbon.

Effect of concentration ratio of TCE to limonene on degradation efficiency of TCE: In abiotic control (Table 1) (treatment with no inoculum and inducer), 15% of TCE was dissipated which might be the result from volatilization of TCE or sorption of TCE on OCIRM.

Table 1: Percentage of TCE removal using different concentration ratios of limonene and concentration of TCE at day 35

	TCE removal (%)			
	R. gordoniae P3		Aerobic sludge	
Concentration ratio (TCE:limonene)	Orange peel	Limonene solution	Orange peel	Limonene solution
1:0	15.95±0.44		15.20±1.09	
1:0.5	23.85±1.25aA	20.04±3.97aA	23.25±1.54aA	29.47±1.78aB
1:1	20.62±4.36aA	18.03±4.82aA	23.16±1.04aA	40.61±2.64bB
1:2.5	25.62±2.27aA	28.05±2.73bA	33.58±1.15bB	41.50±1.93bC
1:5	25.42±2.71aA	31.04±1.05bB	37.14±3.89bC	42.31±2.86bC
Control = 15.29±1.17				

*Comparisons between treatments within each column are significantly different (LSD, $p<0.05$) if marked with different small letters. Comparisons between treatments within each row are significantly different (LSD, $p<0.05$) if marked with different capital letters

Without the presence of OCIRM, the addition of *R. gordoniae* P3 or aerobic sludge did not improve the efficiency of TCE removal which indicated that microorganisms needed oxygen, carbon and especially inducer to degrade TCE under aerobic condition. In contrast, it was obviously seen that limonene in orange peel and limonene solution had significantly improved the degradation of TCE by *R. gordoniae* P3 and aerobic sludge with the removal efficiency in the range of 18.03 to 42.31% (Table 1). The results clearly demonstrated that limonene in orange peel and limonene solution has a potential to be used as the inducer for aerobic co-metabolism of TCE.

The effect of concentration ratio of TCE to limonene in orange peel on TCE removal by *R. gordoniae* P3 was not significant while the highest percentage of TCE removal (31.03%) was obtained when limonene solution was used at the ratio of TCE to limonene solution of 1:5 (Table 1).

Aerobic sludge could remove TCE more efficient than *R. gordoniae* P3. When the orange peel was used as the inducer, the percentages of TCE removal (23.21-23.25%) (Table 1) at the concentration ratio of TCE to limonene in orange peel of 1:0.5 and 1:1 were not significantly different. The increase in limonene concentration in orange peel to the ratio of 1:2.5 significantly improved the TCE degradation by aerobic sludge resulting in the increase in percentage of TCE removal to 33.58%. Further increase in the concentration ratio of TCE to limonene in orange peel to 1:5 did not affect the TCE degradation ability of aerobic sludge suggesting that the concentration ratio of TCE to limonene in orange peel of 1:2.5 was optimal for TCE degradation by aerobic sludge. When limonene solution was used as the inducer, the optimum concentration ratio of TCE to limonene solution was 1:1 indicating by the percentage of TCE removal of 40.61 (Table 1). Increase in concentration of limonene solution to the ratio of 1:2.5 and 1:5 did not increase or worsen the efficiency of TCE removal by aerobic sludge. Limonene solution gave a higher percentage of TCE removal compared to orange peel. These might be due to the fact that limonene in orange peel was inside the orange peel thus it was difficult for microorganisms to access in comparison to limonene solution. When considered that the orange peel is the waste and has no value, it can be a good inducer for TCE remediation process.

The induction of co-metabolic degradation of TCE by various microorganisms using plant limonene and limonene solution had been studied. Suttinun *et al.* (2004) reported that limonene solution was able to induce TCE co-metabolic pathway of *R. gordoniae* P3 resulting in the 10% higher percentage of TCE removal as compared to the treatment without inducer. In addition, lemon oil or lemon grass oil containing approximately 32% of limonene was able to increase TCE removal capability of *Rhodococcus* sp. L4 by 23% in comparison to the treatment without inducer (Suttinun *et al.*, 2009).

Since the concentration ratio of TCE to limonene solution at 1:1 was not significantly different than other ratios and the optimum ratio of TCE to limonene in orange peel was 1:2.5, thus in order to be able to make a comparison between the efficiency of limonene source, the ratio of 1:2.5 was used in the further experiment.

Co-metabolism of TCE: This experiment examined the co-metabolism of TCE by *R. gordoniae* P3 and aerobic sludge in free cells, immobilized cells and biofilm forms using the optimum concentration ratio of TCE to limonene in orange peel and limonene solution of 1:2.5. Percentages of TCE removal after 35 days of incubation were shown in Table 2. When limonene in orange peel was used as the inducer, there was no significantly different among the TCE degradation efficiency of free cell, immobilized cell on corncob and biofilm of inocula (Table 2). The percentage of TCE removal by every forms of *R. gordoniae* P3 were in the range of 31.22-36.99, while the percentage of TCE removal by every forms of aerobic sludge were in the range of 38.93-40.41. Result implied that the efficiency of aerobic sludge to co-metabolite TCE is better than pure culture. The findings were similar to the study of Coyle *et al.* (1993) whom reported that 80-85% of TCE

Table 2: Percentage of TCE removal at day 35 of incubation

	TCE removal (%)	
Treatment	*R. gordoniae* P3	Aerobic sludge
Without inoculum	20.28±2.29a	
Free cell		
Without inducer	15.22±3.81a	16.50±3.78a
Orange peel	31.22±1.24b	38.93±1.54bc
Limonene solution	35.53±3.55bcd	48.61±0.41d
Immobilization		
Without inducer	19.75±2.69a	15.25±3.25a
Orange peel	33.75±0.85bc	39.13±1.23bc
Limonene solution	39.45±2.05cd	47.15±1.63d
Biofilm		
Without inducer	20.40±2.97a	18.82±3.36a
Orange peel	36.99±3.12bcd	40.41±1.97c
Limonene solution	45.81±2.53e	55.89±2.97e

*Comparisons between treatments within each column are significantly different (LSD, $p<0.05$) if marked with different letters

removal could be accomplished by mixed cultures which was greater than that of pure culture, *Psuedomonas putida* (55%) under aerobic condition using phenol as the primary substrate.

When limonene solution was used as the inducer, the TCE removal efficiency by free and immobilized cell forms of the inoculum was not significantly different. Seed inocula in biofilm form showed a better performance of TCE degradation as compared to free and immobilized cell forms with the highest percentage of TCE removal of 45.80 for *R. gordoniae* P3 and 55.89 for aerobic sludge (Table 2). The improvement of TCE degradation by biofilm might be because during the formation of biofilm, some of organic compound such as surfactants have also been produced, which could lead to more bioavailability and better biodegradation of TCE (Singh *et al*., 2006).

Number of TCE degraders i.e., *R. gordoniae* P3 and TCE degrader in aerobic sludge increased from approximately 10^5 cfu mL^{-1} to approximately 10^7 cfu mL^{-1} at 3 days of incubation (Fig. 2). After day 5, number of TCE degraders decreased continuously until the end of incubation. The decrease in number of TCE degraders might result in a decrease in TCE degradation efficiency.

pH of synthetic groundwater increased from 7.0 to 11.0 after 35 days of incubation (Fig. 3) which might inhibit the growth and cause the adverse effect to the TCE degrader. The increase in pH of synthetic groundwater during incubation was due to the release of OH^- from binding cement. The use of the other binding compound which had no effect on pH or the addition of pH maintaining substances may aid the TCE removal ability of microorganisms.

As the result indicated that biofilm form of aerobic sludge is the most efficient inoculum for TCE removal from synthetic groundwater, it was further used to remediate *cis*-DCE, VC and a combination of *cis*-DCE and VC in the next experiment.

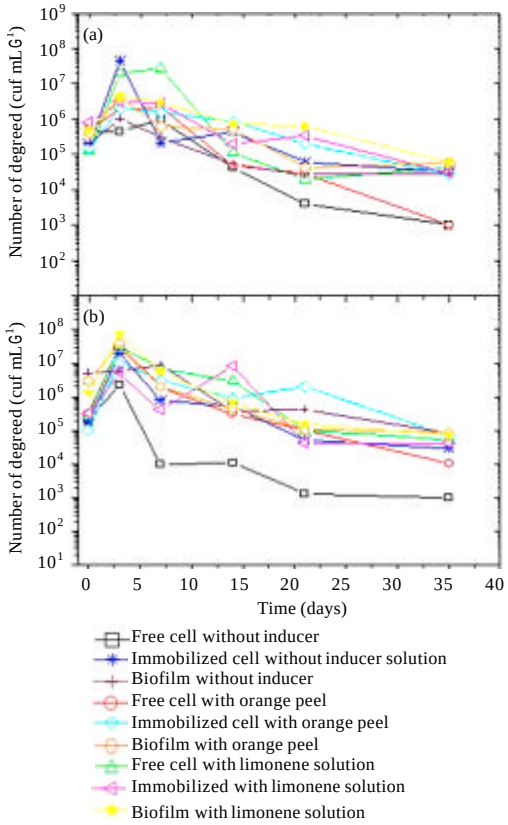

Fig. 2: Cell number of TCE degrader (a) *R. gordoniae* P3 and (b) aerobic sludge

Biodegradation of *cis*-DCE, VC and a combination of *cis*-DCE and VC under aerobic condition: Biodegradation process of *cis*-DCE, VC and a combination of *cis*-DCE and VC by biofilm of aerobic sludge on OCIRM in comparison to free cell was studied. In the abiotic control, 28-34% of *cis*-DCE and VC were degraded but no chloride ions were generated which indicated that some amount of *cis*-DCE and VC were dissipated directly from synthetic groundwater via volatilization process.

The percentage of contaminants removal in the synthetic groundwater was shown in Table 3. The maximum percentage of *cis*-DCE removal of 61.36 was achieved when the biofilm was used as inoculum while the maximum percentage of *cis*-DCE removal was 50.65 when free cell was used, which confirmed that biofilm technique could improve the co-metabolism of TCE and *cis*-DCE.

The maximum percentage of VC removal by the free cell of aerobic sludge and biofilm were 60.69 and 71.89, respectively. Results indicated that the biofilm could degrade VC more efficient than the free cell. It was found that, there was no significantly different among the treatments that had the inducers (limonene from orange peel and limonene solution) and the treatments without

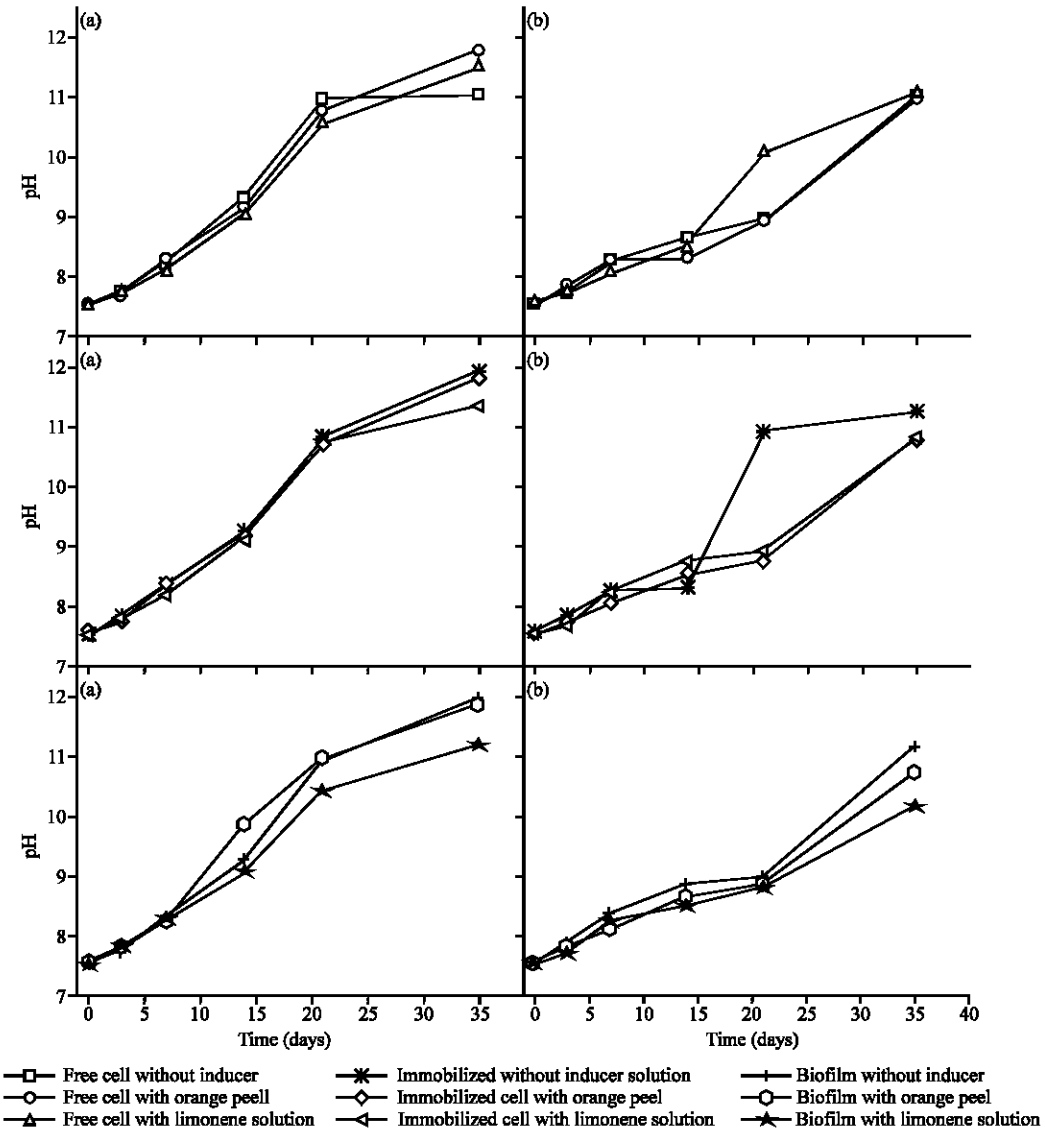

Fig. 3: pH value in microcosm study versus time (a) *R. gordoniae* P3 and (b) aerobic sludge

Table 3: Percentage of contaminants removal in synthetic groundwater containing acclimatized aerobic sludge with different types of organic releasing material after 21 day of incubation

		Biodegradation of *cis*-DCE and VC (%)			
				Combination of *cis*-DCE and VC	
Treatment					
Inoculum	Inducer	*cis*-DCE	VC	*cis*-DCE	VC
Without inoculum	-	28.93±3.17a	33.58±5.76a	33.24±4.38a	34.15±5.46a
Free cel	Without inducer	35.94±2.32b	59.80±3.73b	45.19±5.75b	64.31±4.28b
	Orange peel	48.09±4.21c	60.44±3.90b	55.33±5.81bc	65.14±4.09b
	Limonene solution	50.65±4.70c	60.69±4.02b	56.50±3.72c	65.12±4.22b
Biofilm	Without inducer	37.41±4.41b	68.60±3.11c	46.58±4.11b	75.11±4.77c
	Orange peel	58.35±3.76d	70.02±3.93c	64.59±4.93cd	74.93±4.59c
	Limonene solution	61.36±4.09d	71.89±3.17c	66.14±3.05d	75.67±4.32c

Comparisons between treatments within each column are significantly different (LSD, $p<0.05$) if marked with different letters

inducer which implied that the degradation of VC is not from co-metabolism process which was in correlation to the results in the previous published reports (Freedman *et al*., 2001; Coleman *et al*., 2002; Elango *et al*., 2006).

Biofilm could degrade a combination of *cis*-DCE and VC solution in the synthetic groundwater better than free cell. The maximum percentage of *cis*-DCE and VC removal by biofilm in a combination of *cis*-DCE and VC solution were 66.14 and 75.67, respectively, which were higher than *cis*-DCE and VC removal by free cell (56.50 and 65.12%) (Table 3). Percentage of *cis*-DCE removal in a combination of *cis*-DCE and VC solution was higher than the percentage of *cis*-DCE in treatment of *cis*-DCE alone. Broholm *et al*. (2005) reported that the removal of *cis*-DCE occured concurrently with VC, suggesting that the biodegradation of *cis*-DCE depended on the biodegradation of VC.

cis-DCE and VC were dechlorinated by the inocula resulting in the increase of chloride ion during incubation (Fig. 4a). Chloride ion was found to increase rapidly from day 0 to 7, then gradually decreased. The same phenomena were also observed in a combination of *cis*-DCE and VC solution. The maximum percentage of chloride generated of 32.54, 39.22 and 34.75 were achieved when *cis*-DCE, VC and a combination of *cis*-DCE and VC solution were treated by biofilm when limonene solution was used as inducer.

The variation of pH and cell number of contaminant degraders in the microcosm were monitored as the

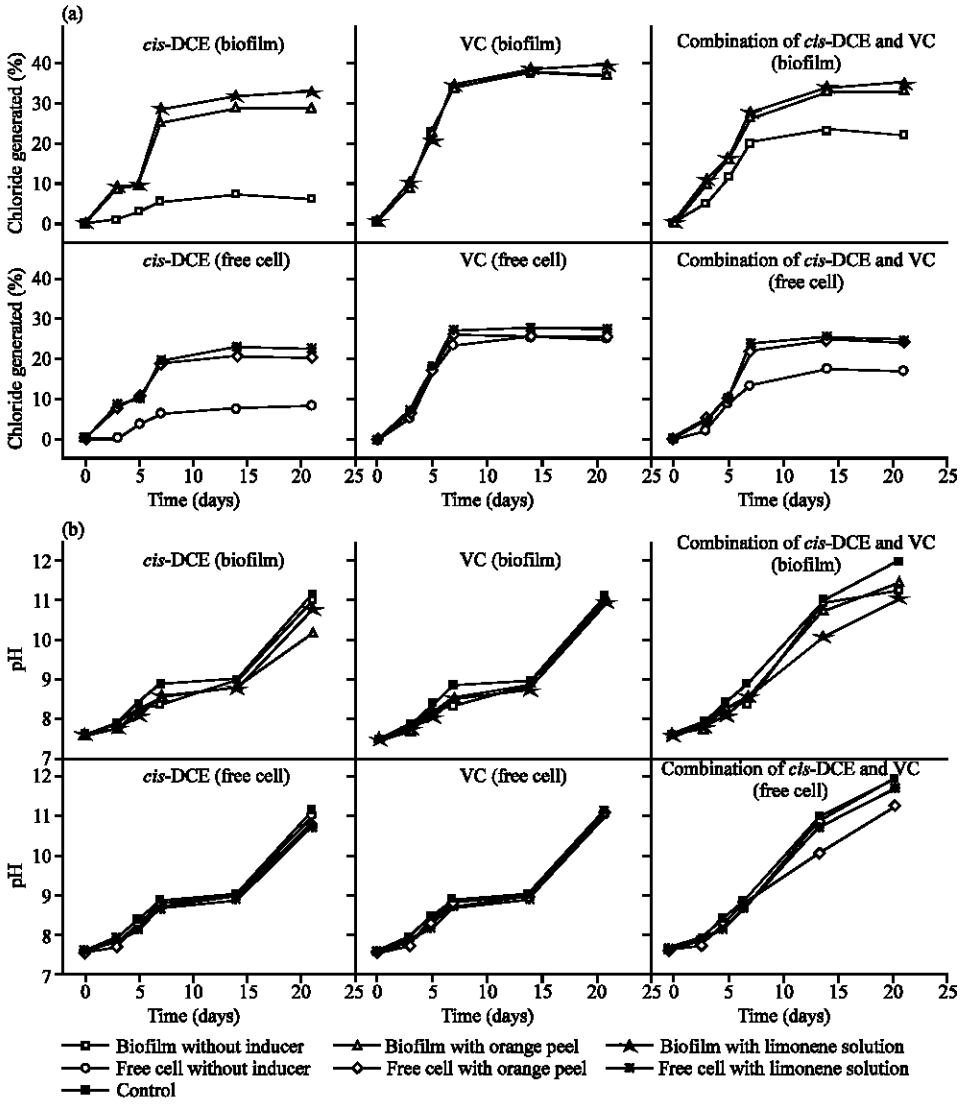

Fig. 4: (a) Percentage of chloride generated and (b) pH in microcosm study

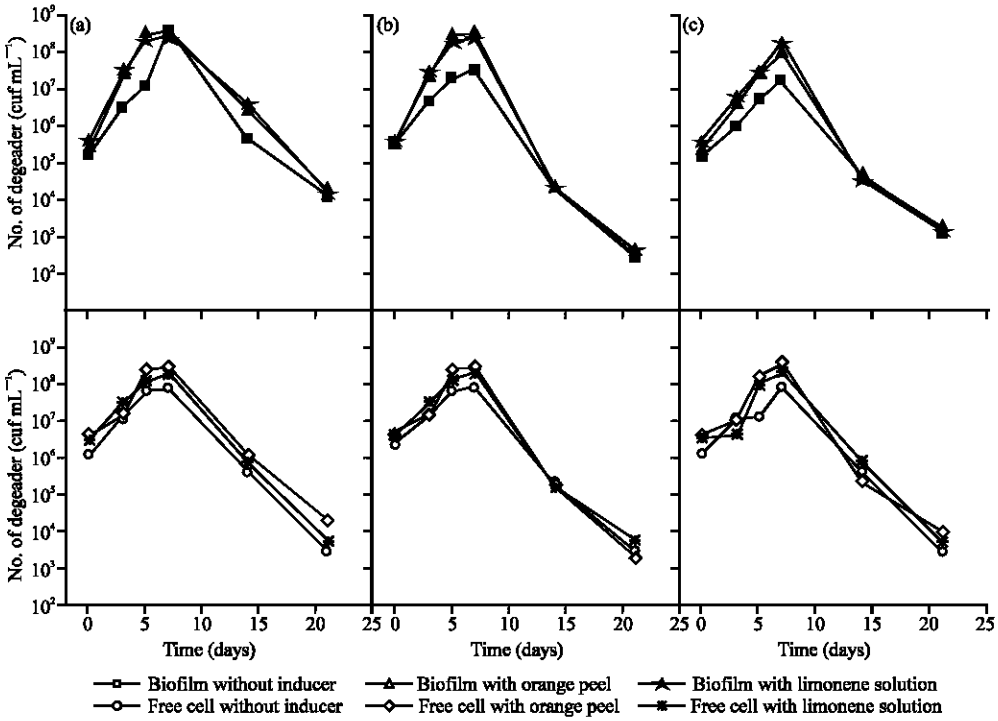

Fig. 5: Cell number of contaminants degrader (a) *cis*-DCE (b) VC and (c) combination of *cis*-DCE and VC solution

important factors affecting the efficiency of *cis*-DCE and VC removal in the microcosm. Initially (day 0-7), pH in microcosm was in the range of 7.5 to 8.5 (Fig. 4b), after that pH increased to approximately 11. These results coincided with the cell number of contaminant degraders in microcosm which initially increased, during day 0-7, from 10^5 to 10^8 cfu mL^{-1} and then decreased over time (Fig. 5) which suggested that an increase in the pH in microcosms caused the adverse effect on the degraders.

CONCLUSIONS

This study successfully constructed the OCIRM to supply oxygen, carbon and inducer for the co-metabolism of TCE and degradation of its metabolites. The optimal size of the OCIRM was 1 cm^3 cube. OCIRM containing sugarcane bagasse as a C source gave a maximum COD releasing rates and DO concentration of 0.30 mg COD day^{-1} g^{-1} cube and 11.95 mg O_2 L^{-1} over 100 days. Limonene in orange peel and limonene solution successfully induce a co-metabolism of TCE and the optimum concentration ratio of TCE to limonene in orange peel and limonene solution was 1:2.5 and 1:1, respectively. Biofilm of *R. gordoniae* P3 and aerobic sludge coated on OCIRM could co-metabolize TCE better than free and immobilized cell forms. *cis*-DCE was effectively degraded by biofilm of aerobic sludge with the presence of limonene as the inducer while VC could be metabolized directly without the addition of inducer. The presence of VC in the system could enhance the degradation of *cis*-DCE by aerobic degrader via co-metabolism process.

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