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Effective bead preparation of coimmobilized methanogenic and methanotrophic bacteria for tetrachloroethene degradation

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Abstract

Three types of coimmobilized methanogenic and methanotrophic bacterial beads – Ca-alginate, Ba-alginate, and Ca-alginate chitosan – were used for tetrachloroethene (PCE) degradation. For the purpose of effective preparation of coimmobilized bacterial beads, the diameter and broken-loading of beads were measured. The activity tests to find the optimal bacteria concentration in the bead were performed. It was found that Ba-alginate beads had superiority in bacterial growth and the degree of strength of beads from the diameter and broken-loading tests. Also, it was shown that it is most effective to add 200 mL of methanogens into 500 mL of 2% alginate solution and 20 mL of methanotrophs into 500 mL to 2% alginate solution. When methanogens and methanotrophs were applied with the Ba-alginate bead in the actual dechlorination of PCE, the biological PCE dechlorination rate was 92%, and there was highly effective degradation of PCE based on the coimmobilized bead. Additionally, relation to the diameter (X) and broken-loading (Y) of the Ba-alginate bead was derived following equation, $Y = 438.02 \exp(-1.4815\,X)$.

Introduction

Chlorinated organic solvents such as tetrachloroethene (perchloroethene, PCE) and trichloroethene (TCE) are contaminated substances that are commonly found in groundwater and contaminated soil (Hirata et al. 1992). Previous methods used to dechlorinate PCE and TCE were methods such as de gassing, adsorption of activated carbon, UV radiation and oxidization using hydrogen peroxide. However, these methods simply work by alternating and accumulating from one medium to another, making it difficult to anticipate the complete degradation of PCE and TCE.

In anaerobic conditions, the biotransformation of PCE and TCE's was observed in nature (Parson et al. 1984) and in laboratories (Vogel & MeCarty 1985; Freedman & Gossett 1989). However, in the case of PCE, single anaerobic treatment using methanogenic bacteria shows a tendency to stop the solution process when a intermediate, DCE, is reached. Plus, treatment by methanotrophic bacteria has a problem of PCE itself not dissolving. Recently, Gerritse et al. (1995) proposed a two stage reactor where the first reactor dechlorinates the PCE and the second intermediates are dissolved by cometabolisim. The proposed two-stage reactor showed a great efficiency in eliminating PCE without the accumulation intermediates.

Method which can simultaneously immobilize in the bead methanogenic bacteria that dechlorinates PCE and methanotrophic bacteria that remove intermediates has advantages in terms of establishing equipment, operations and economy. PCE can be dechlorinated effectively within one reactor by using organisms bead, and organisms can be protected from the external condition. However, in order to degrade PCE by using organisms bead, there are a few tasks that need to be carried out first. First, polymer that does not have an effect on the activity of organisms, and which immobilize organisms for a long period of time needs to be selected. Second, the concentration

of organisms that is coimmobilized in the bead needs to be determined.

The materials used in the encapsulation of organisms can be divided into natural polymer and compound polymer. Agar, agarose, alginate and carrageenan among natural polymers, and polyacryl amide, polystyrene and polyurethane among compound polymers, are mostly used (Kolot 1981). The use of compound polymers has been limited with concern on the activity of organisms due to its virulence, and alginate among natural polymers is widely applied to immobilize various organisms, owing to the immobilization condition that has no restriction, the availability of sodium alginate and easiness of production when compared to other natural polymers (Veliky et al. 1981).

Many different microbial species have been encapsulated in various matrices for different applications. Fungal strains have been encapsulated for biocontrol (Axtell et al. 1987; Fravel et al. 1985) and biodegradation. Encapsulated microalgae have been investigated for metal sequestratioin (Fry et al. 1994) and wastewater nutrient removal (Chevalier et al. 1985). Encapsulated thermophilic (Kanasawud et al. 1989), methanogenic (Heijnen et al. 1988), and other anaerobic bacteria (Karsten 1993) have also been investigated for potential commercial uses. The variety of microorganisms which have been encapsulated successfully and the wide range of applications which have been explored attest to the utility and versatility of this technology and its potential for use in the environment.

In this study, organism beads were produced by adding various consolidating materials (Ca^{2+}, Ba^{2+}, chitosan) to alginate, a basic polymer matrix. The most effective bead and the amount of organisms for immobilization were determined through the activity test of organisms and diameter variation of the bead. Anaerobic methanogens and aerobic methanotrophs were coimmobilized in the bead to degrade the PCE.

Materials and method

Microorganisms

Anaerobic methanogenic bacteria was obtained from an aerobic disester treating municipal wastewaters (Bucheon, Korea). A strain of Methylosinus sporium (ATCC 35069) was grown on copper-free solid low nitrate mineral salt (LNMS) medium (Park et al. 1991) at 30 °C in a mixture of air and methane (1 : 1). The strain was transferred to 1 L flasks and cultivated 4 days at 30 °C on LNMS medium, and the the culture broth was collected, ultrafiltrated, and mixed with the anaerobic sludge.

Ca, Ba-alginate bead production

Mix 20 ml (volume) of methanotrophic bacteria and 200ml (volume) of methanogenic bacteria with 500 ml of 2% (weight/volume) sodium alginate solution. After dropwising the mixed solution into 0.5% (w/v) $CaCl_2$ (Ca-alginate bead) or $BaCl_2$ solution (Ba-alginate bead), agitate at 100 rpm for 3 hours. When beads are formed, rinse them with distilled water and transfer to the reactor. Maintain pH 7–7.5. The above procedure is shown in Figure 1.

Ca-alginate chitosan bead production

Mix the produced Ca-alginate bead into 2% (volume/volume) acetic acid in which 3% (w/v) chitosan is dissolved, as the above method. Agitate the mixed solution at 100 rpm for 5 hours to form chitosan membrane. If Ca-alginate beads are formed, rinse them with distilled water and transfer them to the reactor. Maintain pH 7–7.5.

Measuring the diameter of the bead

In order to test the change in diameter of the bead, 3 types of beads – Ca-alginate, Ba-alginate, and Ca-alginate chitosan – were used. Beads that do not immobilize organisms were also formed, to revise the abiotic change in diameter. Seven beads were randomly picked as samples, and 2 beads with the largest and smallest measurement were removed, and the average diameter was calculated with remaining 5 beads. In order to revise the abiotic change in diameter of the polymer itself, bead that does not immobilize organisms were also measured. The change in diameter of the bead was measured by using Digimatic Caliper (Model No. CD-15CP, Mitutoyo Corp., Japan).

Measuring the broken-loading of bead

In order to measure the broken-loading of bead, 3 types of beads – Ca-alginate, Ba-alginate, and Ca-alginate chitosan bead – were used. Bead that do not immobilize organisms were also formed, to revise the abiotic change in broken-loading, same as when the diameter was measured. Seven beads were randomly

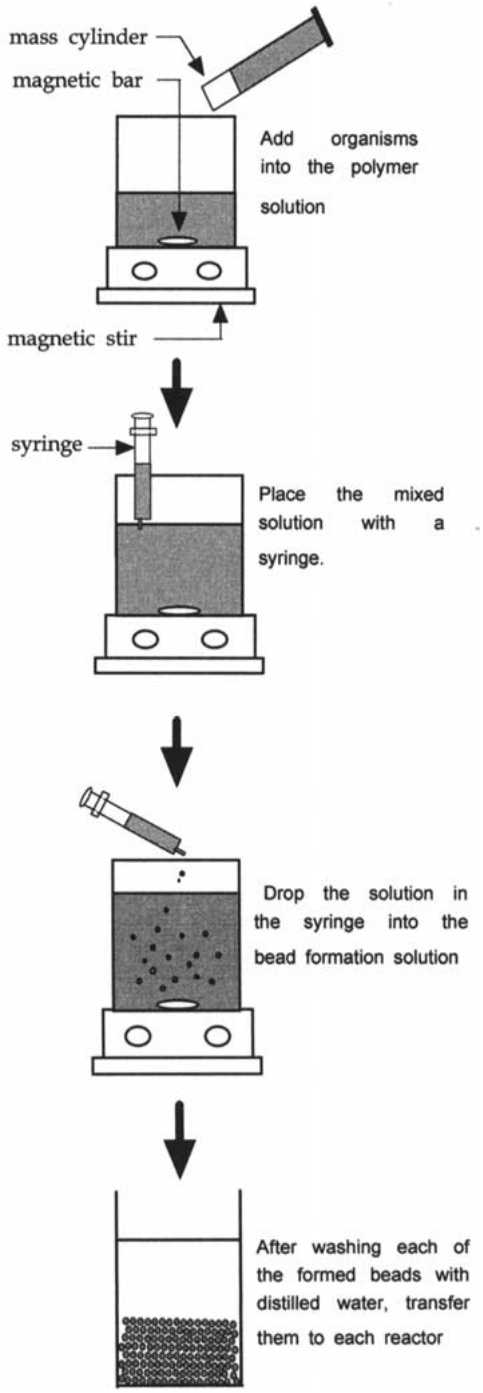

Figure 1. Schematic diagram of Ca- and Ba-alginate bead production.

picked as samples, and the average broken-loading was calculated. Broken-loading was defined with external load the caused the bead to start breaking. In other words, how much weight was needed to break a bead. The change in broken-loading of the bead was measured by using Texture Analyzer (Taxt; 2/25, Stable Micro system, Japan)

Organisms activity test of methanogens single immobilization bead

Single immobilization bead, different from the volume of immobilized methanogenic bacteria, was produced, to determine the amount of optimum methanogens (150, 200, 250, and 300 mL of methanogens were each added to 500 mL of 2% alginate solution). The initial concentration of acetic acid, the substrate of this test, was 600 mg/L. Serum bottles of 120 mL was used for the reactors, and 2 g (net weight) of Ba-alginate bead and 20 mL of medium that has been autoclaved for 20 minutes at 121 °C were injected into each serum bottle. The formation of medium that has been put in, is shown in Table 1. The pH of medium was maintained at 7.0–7.2, and the test was conducted by agitation at 100 rpm, by using a mixer at anaerobic condition of 25 °C. The change in protein concentration and substrate concentration of the bead were measured every 6 hours, and the amount of optimum methagonens was determined.

Organisms activity test of methanotrophs single immobilization bead

In order to determine the amount of optimum methanotrophs, single immobilization bead, different from the volume of immobilized methanotrophs bacteria, was produced (15, 20, 25, and 30 mL of methanotrophs were each added to 500 mL of 2% alginate solution). Serum bottles of 120 mL were used for the test, and 2 g of Ba-alginate bead immobilized with methanotrophs only, and 20 mL of LNMS medium (Park et al. 1991) were injected into each serum bottle. Methane-air gas with 25% (v/v) concentration of methane, the substrate of this test, was injected into the serum bottle at the speed of current of 128 mL/min. Right after the injection, it was shook intensely and after it had been stopped, sample ore was collected from the headspace and the concentration measured with the GC was determined as the initial concentration. For the facility of the transmission of methane, agitation was conducted with a mixer at 25 °C, and pH 7.0–7.2 was maintained.

Table 1. Composition of medium

Composition per liter	
KH_2PO_4	1.36 g
NH_4HCO_3	0.79 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$CaCl_2$	0.2 g
Yeast extract	0.025 g
CH_3COOH	1.5 mL
Trace element solution	1 mL
pH 7.0 ± 0.2 at 25° C	
Trace element solution Composition per liter	
EDTA	1 g
$FeSO_4 \cdot 7H_2O$	2 g
H_3BO_3	300 mg
$CoCl_2 \cdot 6H_2O$	200 mg
$ZnSO_4$ $7H_2O$	100 mg
$MnCl_2 \cdot 4H_2O$	30 mg
$Na_2MoO_4 \cdot 2H_2O$	30 mg
$NiCl_2 \cdot 6H_2O$	20 mg
$CuCl_2 \cdot 2H_2O$	10 mg

The test was conducted. The change in protein concentration and substrate concentration of the bead were measured every 6 hours, and the amount of optimum methanotrophs was determined.

Reactor setup and operation

The experiment was operated by injecting a coimmobilized Ba^{2+}-alginate bead using methanogenic bacteria and methanotrophic bacteria in a continuous reactor. The Ba^{2+}-alginate bead was produced by injecting methanogenic bacteria and methanotrophic bacteria at a ratio of 200 : 20 mL. A total of 150 g of bead and culture were injected into the reactor to bring the total working volume of the reactor to 1.65 L. The culture was used after autoclaving for 20 minutes at 121° C and the pH was kept at 7.0 ± 0.2. The culture was injected into a 2 ml/min reactor. The media was made by adding the following substances to 1 L of distilled water: substrate 2500 mg/L as COD; KH_2PO_4 1.36 g; NH_4HCO_3 0.79 g; $MgSO_4 \cdot 7H_2O$ 0.1 g; $CaCl_2$ 0.2 g; yeast extract 0.025 g; trace element solution 1 mL. The trace element solution consists of the following (in mg/L): EDTA 1000; $FeSO_4 \cdot 7H_2O$ 2000; H_3BO_3 300; $CoCl_2 \cdot 6H_2O$ 200; $ZnSO_4 \cdot 7H_2O$ 100; $MnCl_2 \cdot 4H_2O$ 30; $Na_2MoO_4 \cdot 2H_2O$ 30; $NiCl_2 \cdot 6H_2O$ 20; $CuCl_2 \cdot 2H_2O$ 10. Reactor was operated at HRT 17h and the temperature at 30° C, with enforced stirring. The reactor's diagram is listed in Figure 2.

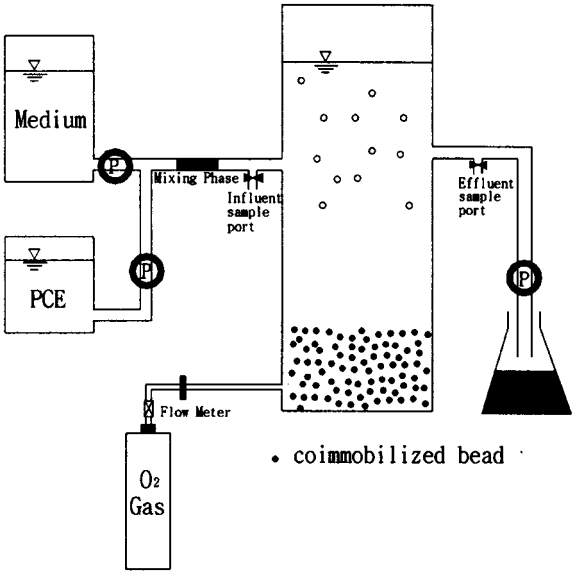

Figure 2. Schemetic of coimmobilized bead reactor.

Abiotic test was also conducted to closely examine the loss of abiotic PCE based on the adsorption of bead or volatilization. One more set of continuous reactors with the same conditions were produced, and Ba-alginate bead in which the organisms are not immobilized were injected into the reactor, and the abiotic conversion rate of PCE was observed.

Chemicals and analytical method

Sodium alginate and chitosan were purchased from Junsei Chemical Corp., Japan. The chlorinated organic compounds used in the study were PCE (99.7%; Merck, Germany). All other chemicals were reagent grade.

Protein contents of beads were estimated using the modified lowry method (Protein assay kit, Sigma chemical corp, USA). PCE was measured by liquid-liquid extraction using ECD detector equipped with GC (HP 6890+, USA). Extraction solvent used was n-Hexane (99+%, Sigma, capilly GC grade, USA). When a boundary layer developed after filling a 2 ml vial with solution made up of the sample and extraction solvent with a ratio of 1 : 10, 1 ul was extracted from the solvent layer and injected into the GC. To establish a line to measure the concentration of PCE, 100 ug/ml of standard solvent (Chem Service, USA) was used. The detailed GC operation conditions are

Table 2. GC operation conditions for measuring PCE

	GC operation		Conditions
Inlet	Carrier gas		N_2
	Temp.		200° C
	Pressure		11.31 psi
	Split ratio		20 : 1
	Injection volume		1 ul
Column	Type		DB-624 (Agilent Tech., USA)
	Length		30 m
	Inner diameter		0.32 mm
	Film thickness		0.18 um
Oven	Temp.	starting	80° C
		increasing rate	10° C/min
Detector	Type		ECD
	Temp.		300° C
	Make-up		N_2

shown in Table 2. Also, acetate and methane in beads were determined by gas chromatography (HP 6890+, USA).

Results and discussion

Change in diameter of bead

As organisms inhabit in a restricted space of bead, the change in diameter of bead can be used as indirect index of organisms growth. The diameter of Ca-alginate bead greatly increased due to rapid growth, 6 hours after production, in the case of both bead-bead with immobilized organisms and bead with non-immobilized organisms. After showing slow increase after 12 hours, the bead was broken in about 42 hours. In the case of bead with immobilized organisms, the bead that had a diameter of 3.5 mm increased until 5.78 mm, and in the case of bead with non-immobilized organisms, the diameter changed from 3.5 to 5.12 mm.

In the case of Ba-alginate bead, there was great difference between the bead that are not immobilized and bead with non-immobilized organisms. Bead with non-immobilized organisms continued longer than the Ca-alginate bead or Ca-alginate chitosan bead, and broke after about 300 hours. Contrary to this, Ba-alginate bead with immobilized organisms had the tendency to break after about 120 hours. In the

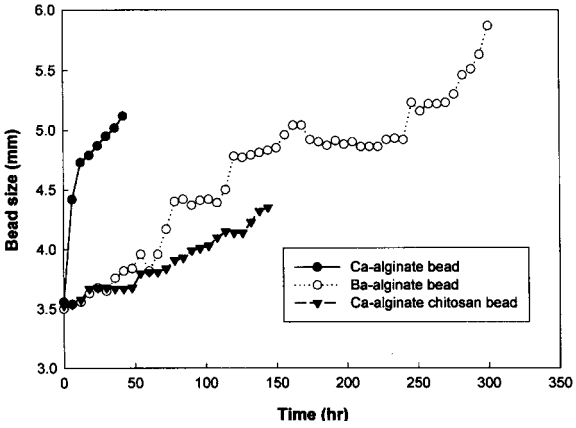

Figure 3. Change in diameter of beads with non-immobilized organisms.

case of Ba-alginate bead with immobilized organisms, the diameter changed from 3.5 to 5.94 mm, and Ba-alginate bead with non-immobilized organisms changed from 3.5 to 5.87 mm. Ba-alginate bead with non-immobilized organisms broke after a longer period of time than Ba-alginate bead with immobilized organisms, and this is judged to be based on the existence of organisms in the bead.

The change in diameter in the case of Ca-alginate bead increased most slowly when compared to the other two types of bead. However, both the bead with immobilized organisms and non-immobilized organisms showed similar change in diameter. Based on such results, it is judged that organisms do not grow well in Ca-alginate chitosan bead. Both of the Ca-alginate chitosan bead with immobilized organisms and non-immobilized organisms broke in 150 hours and when the bead broke, the diameter of both of the bead were 4.35 mm. The above-mentioned results are shown in Figure 3 and 4.

Broken-loading of bead

The broken-loading of bead is an important guide post that shows how long organisms can be immobilized stably. In the case of Ca-alginate bead, there was a decrease in rapid broken-loading after 6 hours in both the bead with immobilized organisms and non-immobilized organisms, and there was slow reduction after that. They were all broken after 42 hours. As for Ca-alginate bead, both the bead with immobilized organisms and non-immobilized organisms broke down more rapidly than Ba-alginate bead or Ca-alginate chitosan bead (they broke after 42 hours of test). I believe that it was not because of the growth of or-

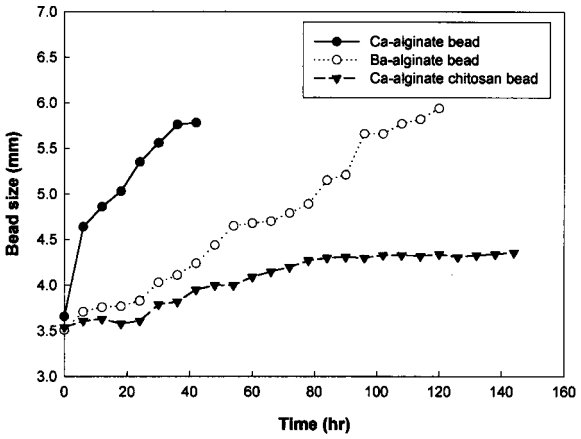

Figure 4. Change in diameter of beads with immobilized organisms.

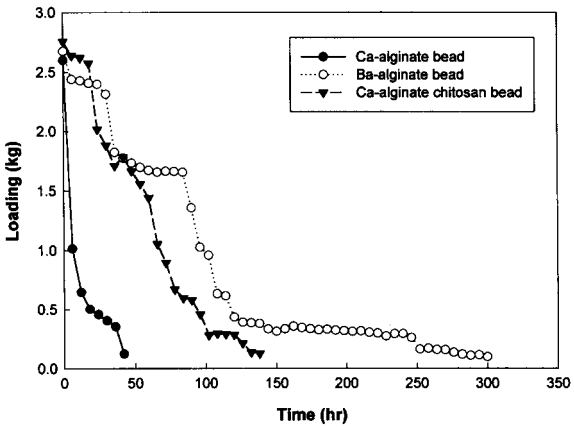

Figure 5. Change in broken-loading of beads with non-immobilized organisms.

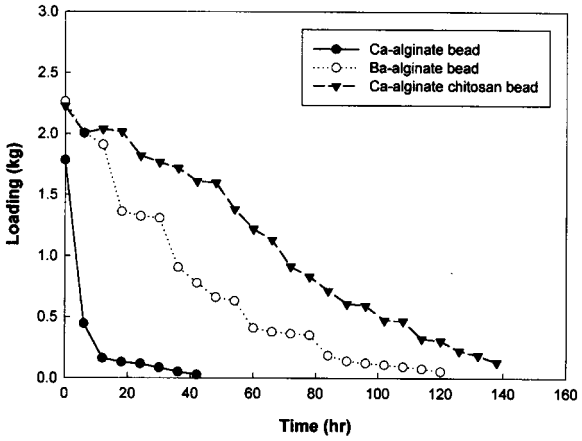

Figure 6. Change in broken-loading of beads with immobilized organisms.

ganisms but because the bead got weaker as the Ca^{2+} ion got eluted by the phosphate buffer.

Ba-alginate bead with immobilized organisms showed change in rapid broken-loading in short period of time when compared to Ba-alginate bead with non-immobilized organisms. This is judged to be caused by the growth of organisms in a restricted area, which rapidly reduced the broken-loading of the bead. Ba-alginate bead with immobilized organisms may have broke in a shorter period of time than Ba-alginate bead with non-immobilized organisms, but it took 120 hours – it continued on longer when compared to Ca-alginate bead.

In the case of Ca-alginate chitosan bead, both the bead with immobilized organisms and non-immobilized organisms broke after 138 hours, and the change in broken-loading was slow. The change in broken-loading of both Ca-alginate chitosan bead with immobilized organisms and non-immobilized organisms is not significantly different, as the long period of time of chitosan coating effected the activity of the organisms, or the chitosan coating membrane prevented the transmission of oxygen and substrate to the organisms as it was encapsulated in the bead.

Generalizing the above results, the bead that can immobilize organisms most stably and for the longest period of time is concluded to be the Ba-alginate bead. The above results are shown in Figure 5 and 6.

Activity test of methanogens in bead

As it can be seen in Figure 7, the protein concentration of single immobilized bead of all concentration gradually decreases until about 42 hours, and increases after that. It is because organisms go through a phase of "habitat segregation" (Kurosawa et al. 1990), in order to adapt and live in a new habitat- the bead. Methanogens are immobilized throughout the whole area of the bead. Only the organisms that are in the inner zone of the bead, the absolute anaerobic zone, during the period of habitat segregation can survive and grow. Most of the other organisms that are immobilized on the surface of the bead die out. There needs to be at least 42–48 hours of acclimation period, for the period of habitat segregation – the period to grow by being located in the inner zone of the bead. And, during this period of time, the protein concentration of organisms decreases. The concentration of organisms gradually increased in all single immobilized beads that have gone through the acclimation period, and there was a leakage of organisms in the entire bead

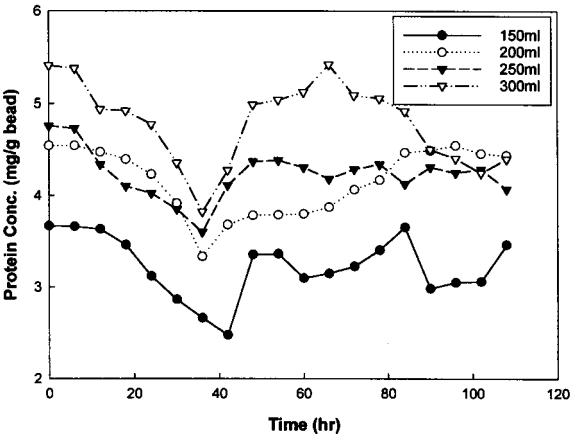

Figure 7. Change in protein concentration in beads with immobilized methanogens.

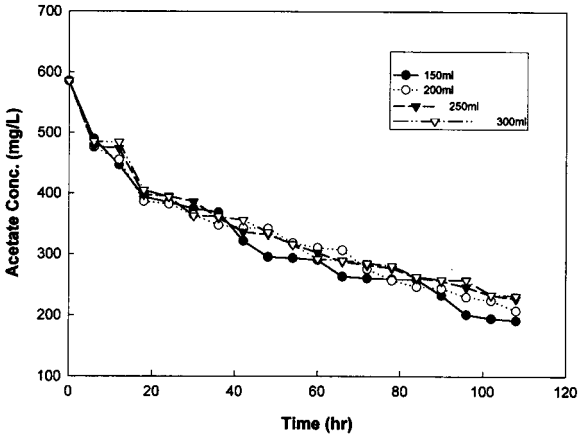

Figure 8. Change in acetate concentration in beads with immobilized methanogens.

after 54 hours. In particular, there was great leakage in bead that had many organisms of 300 mL. Such leakage of organisms in bead occurred because the gel matrix of the bead possessed cell entrapment limits. Organisms entrapped in bead were of greater concentration than the amount the gel matrix can entrap, or if the organisms grow exceeding the entrapment concentration after habitat segregation, the organisms that exceeds the limited concentration leaks out through the openings in bead.

If the amount of methanogens is 300 mL, the leakage that occurred was the fastest at 12 hours, due to the initial high concentration of organisms, and if the organisms grew exceeding the amount the gel matrix of bead can entrap after going through acclimation period, a large amount of organisms leaked out through the openings in bead. After 300 hours, the concentration of organisms was the lowest at 2.84 mg/g bead.

Bead with 150 mL of single immobilized methanogens maintained a low concentration of organisms on the whole. And bead with 250 mL of single immobilized methanogens, maintain a fixed concentration of organisms after the acclimation period, and there were no great leakages of organisms but after 300 hours, there was low concentration of organisms of 200 mL – it was lower than the single immobilized bead. Bead with 200 mL of single immobilized methanogens gradually increased in concentration of organisms after the acclimation period, and after 84 hours, the protein concentration of organisms was maintained at 4.45 mg/g bead until the point of time when the bead broke.

In the bead with different amounts of immobilized organisms, the substrate consumption amount was regular in nearly all beads. This is because the concentration of organisms that can grow in bead is restricted, as bead possesses cell entrapment limits. Consequently, the concentration of organisms that can survive in bead – with no relation to the immobilized amount of initial organisms – is nearly of the same level in all beads. Therefore, the consumed amount of substrate is similar in all beads.

Through the change in substrate consumption and protein concentration of organisms, it can be seen that it is most effective to add 200 mL of methanogens into 500 mL of 2% alginate solution. The above results are shown in Figure 7 and 8.

Activity test of methanotrophs in bead

As it is shown in Figure 9, the protein concentration gradually decreased until about 12 hours then increased in the entire bead with different amounts of organisms. As it is the same in case of methanogens, it is because organisms go through a phase of "habitat segregation" in order to adapt and live in a new habitat. The concentration of organisms in bead with immobilized methanotrophs that have passed the phase of habitat segregation gradually increased, but there was slight leakage observed in all bead after 36 hours. The organisms that have grown, exceeding the amount of organisms that the bead- that have passed the phase of habitat segregation- can entrap, leaks out of the openings of bead. There was the most leakage of organisms in the bead with 30 mL of immobilized methanotrophs, and bead with 25 mL of immobilized

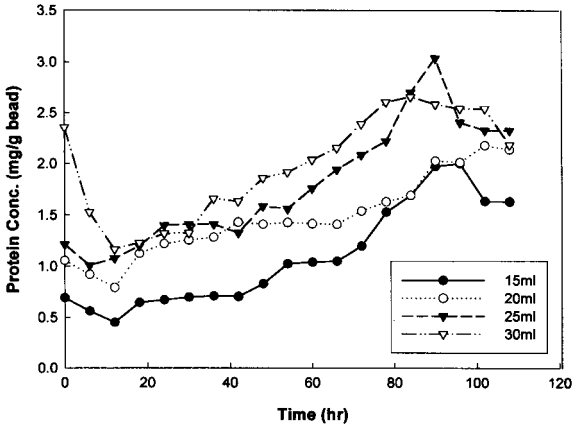

Figure 9. Change in protein concentration in beads with immobilized methanotrophs.

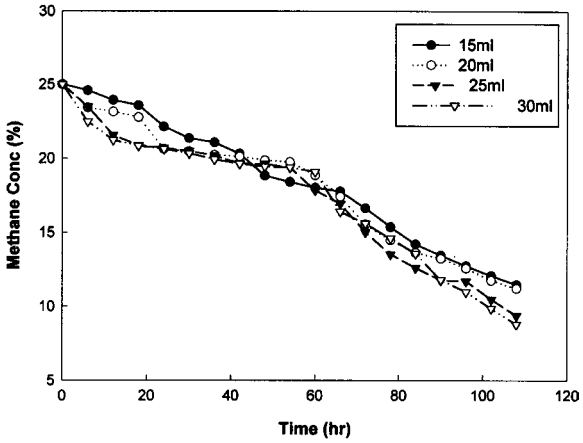

Figure 10. Change in methane concentration in beads with immobilized methanotrophs.

methanotrophs could not maintain the concentration of organisms after 300 hours. In addition, the protein concentration of bead with 15 mL of immobilized methanotrophs was lower than the bead with other amount of immobilized methanotrophs. There was the lowest leakage of organisms in bead with 20 mL of immobilized methanotrophs, and after the acclimation period, the concentration of organisms continually increased without any decrease. Then after 90 hours, the concentration of organisms was maintained for about 300 hours at 2.1 mg protein/g bead.

There was regular amount of substrate consumption in the entire bead with different amounts of immobilized methanotrophs. This is because the concentration of organisms that can grow in bead is restricted, as bead possesses cell entrapment limits, as it is the case for bead with immobilized methanogens. Through the change in substrate consumption and protein concentration of methanotrophs, it can be seen that it is most effective to add 20 mL of methanotrophs into 500 mL of 2% alginate solution. The above results are shown in Figure 9 and 10.

PCE dechlorination

Figure 11 shows the PCE concentration of the number of flow according to time. When only PCE, not bead, is injected, the PCE concentration slightly decreases from the initial PCE concentration. It is because of the effect on the volatilization of PCE, as PCE is in the reactor medium only. The average rate of PCE volatilization was 6.2%. When bead with non-immobilized organisms were injected, there was a greater reduction gap in the concentration of PCE in the reactor

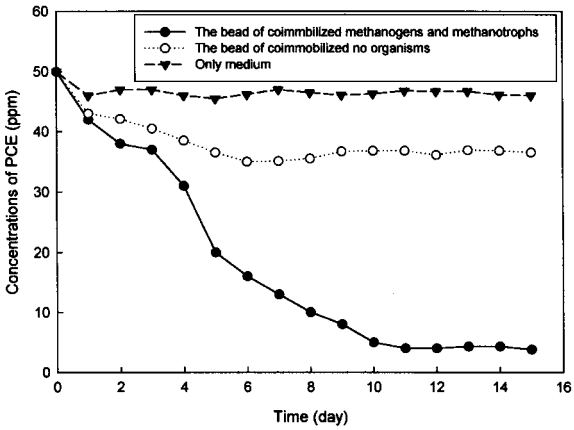

Figure 11. Concentration of PCE in effluent.

without bead. This is because the PCE gets physically absorbed on the bead surface. The average concentration of adsorbed PCE is 26.5% even though the effect of volatilization is excluded; around 20% of PCE gets physically absorbed in the bead. In the reactor where Ba-alginate bead with coimmobilized methanogens and methanotrophs is injected, there was rapid reduction of PCE in the initial period; the final dechlorination rate was 92%. The great reduction of PCE concentration in the initial period is caused by the volatilization of PCE and its physical adsorption. However, the absorbed PCE degrades by the function of organisms that are immobilized in the bead. The initial concentration of 50 ppm decreased to 3.8 ppm. Judging from the above results, Ba-alginate bead with optimum coimmobilized methanogens and methanotrophs effectively dechlorinates PCE.

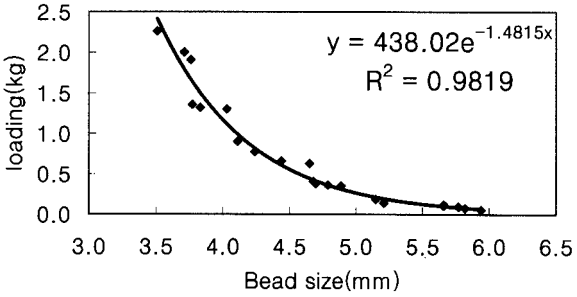

Figure 12. The relation between broken-loading and diameter of bead.

The relation between broken-loading and diameter of bead

The relation between broken-loading and diameter of bead was derived through tests on broken-loading and change in diameter with the Ba-alginate bead that has coimmobilized 20 mL of methanotrophs and 200 mL of methanogens in 500 mL of 2% alginate solution, the most effective bead. This relation showed exponential function of the reduction of broken-loading according to the increase in the bead's diameter. It is as follows: Y = 438.02 exp(–1.4815 X); [where Y is the broken-loading (Kg); X is the diameter of bead (mm)].

Conclusions

As the result of testing the broken-loading that shows the strength of bead and the change in diameter of bead – the indirect guide post of growth of organisms – Ba-alginate bead showed the best results. As the result of testing the activity of the bead with single immobilized methanogens and methanotrophs, the optimum amount of immobilized organisms was adding 200 mL of methanogens and 20 mL of methanotrophs to 500 mL of 2% alginate solution. When methanogens and methanotrophs were applied with the Ba-alginate bead in the actual dechlorination of PCE, the biological PCE dechlorination rate was 92%, and there was highly effective degradation of PCE based on the coimmobilized bead. The following relation was formed between the diameter of bead (X, mm) and the broken-loading (Y, Kg), as the change in broken-loading and diameter of the Ba-alginate bead. Y = 438.02 exp(–1.4815 X). The relation between the diameter and broken-loading can be deduced from this method.

Acknowledgement

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