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Biodegradation analyses of trichloroethylene (TCE) by bacteria and its use for biosensing of TCE

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ABSTRACT

Trichloroethylene (TCE) is a toxic, recalcitrant groundwater pollutant. TCE-degrading microorganisms were isolated from various environments. The aerobic bacteria isolated from toluene- and tryptophan-containing media were *Pseudomonas* sp. strain ASA86 and *Burkholderia* sp. strain TAM17, respectively; these are necessary for inducing TCE biodegradation in a selective medium. The half-degradation time of TCE to a concentration of 1 mg/L was 18 h for strain ASA86 and 7 days for strain TAM17. While identifying toluene/TCE degradation genes, we found that in strain ASA86, the gene was the same as the *todC1* gene product encoding toluene dioxygenase identified in *Pseudomonas putida* F1, and that in strain TAM17, the gene was similar to the *tecA1* gene product encoding chlorobenzene dioxygenase identified in *Burkholderia* sp. PS12. A novel TCE biosensor was developed using strain ASA86 as the inducer of toluene under aerobic conditions. The TCE biosensor exhibited a linear relationship below 3 ppm TCE. Detection limit of the biosensor was 0.05 ppm TCE. The response time of the biosensor was less than 10 min. The biosensor response displayed a constant level during a 2 day period. The TCE biosensor displayed sufficient sensitivity for monitoring TCE in environmental systems.

1. Introduction

Trichloroethylene (TCE) is a volatile chlorinated aliphatic compound that is extensively used as an organic solvent, a dry cleaning fluid, and a degreaser in industrial processes. TCE reaches groundwater aquifers from the soils of disposal sites, and remains as a soil and groundwater contaminant [1–4]. TCE is one of the many recalcitrant pollutants in environmental systems. Consortia of anaerobic bacteria degrade TCE to vinyl chloride, a highly toxic chemical [5] and a known human carcinogen [6], by reductive dehalogenation. On the other hand, aerobic bacteria can biodegrade TCE without forming any undesirable by-products. Many microorganisms [7–13] have been isolated from hazardous sites. Recently, bioremediation was carried out to remove TCE because it is a widespread soil and groundwater pollutant and is a potential threat of human health [14–18]. TCE is usually degraded by aerobic cometabolism, which requires the presence of a cosubstrate such as methane, ammonia, or toluene-containing aromatic compounds [19]. Aerobic, cometabolic degradation of TCE is mediated by oxygenase enzymes such as soluble methane monooxygenases of *Methylosinus trichosporium* OB3b [20], toluene monooxygenases of *Burkholderia cepacia* G4 [9] and *Pseudomonas mendocina* KR1 [21], and toluene dioxygenases of *Pseudomonas putida* F1 [13]. Thus, microorganisms require oxygen as a cosubstrate for oxygenase for the aerobic cooxidation of TCE.

TCE is major pollutant of surface, ground, and drinking water. Many studies have focused on eliminating TCE pollution in environmental systems [15,17,18]. The determination of volatile organic compounds in water strongly demands accurate and precise chemical analysis. For this purpose, many analytical methods have been developed and are currently in use [22–25]. However, in addition to expensive equipments, these methods involve lengthy sampling times and preparatory steps. To overcome these drawbacks, microbial biosensors that enable simple and quick determination of various organic pollutants in environmental systems, have been developed [26]. However, biosensors for the determination of TCE have been reported only in a few publications; they have used either gene recombination [27,28] or redox mediators [29].

In this study, we described the screening of TCE-degrading microorganisms from various environments, and the characterization of TCE bioavailability and biodegradation to organic compounds. In addition, a novel microbial biosensor was developed for the evaluation of TCE; it used *Pseudomonas* sp. strain ASA86, which is characterized as a bioelement.

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2. Experimental

2.1. Chemicals

TCE (99% purity) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Chemicals for preparation of media and analyses were obtained from Wako Pure Chemical Industries, Ltd., Sigma Chemical Co. (Milwaukee, WI) and Difco Laboratories (Detroit, MI).

2.2. Media

The medium used for isolating TCE-degrading microorganisms had the following composition per liter of distilled water: $NaNO_3$, 2.2 g; NaH_2PO_4, 0.2 g; Na_2HPO_4, 0.4 g; $MgSO_4$, 0.2 g; KCl, 0.04 g; $CaCl_2$, 0.02 g; $FeSO_4{\cdot}7H_2O$, 1 mg; MoO_3, 10 μg; $CuSO_4{\cdot}5H_2O$, 5 μg; H_3BO_3, 10 μg; $MnSO_4{\cdot}5H_2O$, 10 μg; $ZnSO_4{\cdot}7H_2O$, 7 μg; biotin, 2 μg; folic acid, 2 μg; pyridoxine hydrochloride, 10 μg; thiamine hydrochloride, 5 μg; riboflavin, 5 μg; nicotinic acid, 5 μg; DL-calcium pantothenate, 5 μg; vitamin B12, 0.1 μg; *p*-aminobenzoic acid, 5 μg; and lipoic acid, 5 μg. The pH of the medium was 7.0. Then, 0.2 g toluene or tryptophan was added as carbon sources in the medium described above. Toluene- and tryptophan-containing media were prepared, respectively. For the solid medium, 1.0% agarose was added.

2.3. Screening of TCE-degrading microorganisms

Many soil and activated sludge samples were collected and screened for their ability to degrade TCE. Soil (1.0 g) or activated sludge (2 mL) was added to a 70- mL serum vial containing 20 mL of each medium, followed by the addition of 1 mg/L TCE to each vial. The serum vials were sealed with butyl stoppers or silver-coated corks, which were crimped with aluminum caps. Vials were then cultured for 14 days at 30 °C with shaking at 170 rpm. Subculturing was carried out every week; each transfer involved the addition of 0.2 mL of broth to 20 mL fresh medium. This procedure was repeated for a total of three transfers. The final liquid culture was streaked onto agarose plates. The agarose plates were incubated for 2–3 days at 30 °C. Isolated colonies were picked and purified by restreaking 3 times, and were tested in liquid culture for their ability to utilize toluene or tryptophan as carbon sources.

Taxonomic tests were carried out by Gram staining, checking for motility, and determining morphological and physiological characteristics (API20NE kit, bioMérieux, Marcy), G + C content [30]. Cell growth was measured by determining optical density (OD) at 660 nm using a spectrophotometer. The increase in OD_{660} over the incubation period was used to estimate whether the isolate was able to grow on toluene or tryptophan.

2.4. Biodegradation assays

TCE degradation was evaluated by measuring the TCE concentration in the headspace gas of the serum vial by gas chromatography. TCE was separated between the liquid and gas phases in the serum vial, and as per Henry's law, the headspace TCE concentration was proportional to the liquid concentration.

Gas chromatography analysis was conducted by using a HITACHI G-3000 gas chromatograph (HITACHI, Tokyo) equipped with a flame ionization detector. Separation was effected onto a glass column (length, 3 m; external diameter, 3 mm; internal diameter, 1.8 mm) packed with silicone DC-550 (24% phenyl methyl silicone). The operating conditions were as follows: sample volume, 100 μL; carrier gas, 30 mL/min of nitrogen; injector temperature, 200 °C; detector temperature, 200 °C; oven temperature, 80 °C. Headspace samples were periodically collected using gastight syringes (Hamilton, Reno, NV), and were directly injected into the gas chromatograph. Analysis of toluene was performed using the procedure described above for TCE assays. Chloride ions released by TCE biodegradation, were determined by the mercuric thiocyanate method according to the manufacturer's protocol (DR2000 spectrophotometer, HACH Co, Loveland, CO).

2.5. Preparation of genomic DNA and plasmids

The isolated strains were grown at 30 °C in a medium with either toluene or tryptophan as the carbon source. Genomic DNA of each strain was prepared from a stationary phase culture. Cell pellets were washed and resuspended in 0.5 mL of lysis buffer [30 mM Tris–HCl (pH 7.4), 100 mM NaCl, and 5 mM EDTA], followed by the addition of 0.1 mg lysozyme and 50 μg RNase A; pellets were then incubated at 37 °C for 10 min. Lysis was performed by the addition of 0.1 mg proteinase K and sodium dodecyl sulfate (SDS) to a final concentration of 1%, and incubation at 37 °C for 45 min. The suspension was repeatedly extracted with an equal volume of phenol-CIA (25:24:1 of phenol:chloroform:isoamyl alcohol) until there was a clear interface upon centrifugation, followed by ethanol precipitation in the presence of 3 M NaOAc (pH 5.2). The pellets were washed with cold 75% ethanol, air-dried and dissolved in TE [10 mM Tris–HCl (pH 7.4) and 1 mM EDTA]. The quality and quantity of the extracted DNA were checked by measuring the UV absorption spectrum (Beckman DU-7000 spectrophotometer, Beckman, Brea, CA) [31].

Escherichia coli DH5α was routinely cultured in Luria-Bertani medium at 37 °C. *E. coli* DH5α harboring TA vector (Invitrogen, Carlsbad, CA) was grown in a selective medium containing ampicillin (100 μg/mL). Plasmid minipreps were performed using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI). DNA purification was performed with the Wizard DNA Purification Kit (Promega).

2.6. PCR and DNA sequence analysis

DNA fragments of the *todC1* gene [32] were amplified by using primers 5′-CCTGAACAAGAAGGAATGGAGCC-3′ (positions 1042–1064) and 5′-TGATACCTGGGAGGAAGGAACAC-3′ (positions 1592–1570). PCRs were performed with 2 U of *Ex Taq*™ DNA polymerase (Takara, Shiga), with 0.2 mM of each primer set and 100 ng of DNA template in a final volume of 100 μL. PCRs were carried out on a GeneAmp PCR System 9700 (Perkin-Elmer Inc., Foster, CA). PCR conditions were as follows: 1 cycle at 94 °C for 5 min; 25 cycles at 94 °C for 30 s, at 55 °C for 30 s, and at 72 °C for 1 min/kb; and 1 cycle at 72 °C for 7 min. PCR products were analyzed by electrophoresis, and then cloned into TA cloning vectors according to the manufacturer's protocol (Invitrogen).

The DNA sequence was determined using the dRhodamine (dichlororhodamine) Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The reaction products were analyzed by an ABI PRISM 377 DNA Sequencer (Perkin-Elmer Inc.). Similarity searches were performed using the BLAST program and GenBank databases. Nucleotide sequence alignments were performed using the CLUSTAL W program [33].

2.7. Construction of biosensor system for TCE determination

Cells in stationary phase were harvested by centrifugation at 6000 rpm for 10 min, washed twice with 10 mM phosphate buffer (pH 7.0), and were subsequently resuspended in the same buffer. The biofilm was prepared using an aspirator connected to a syringe filter holder (Advantec, Tokyo). A suspension of 1 mL (70 mg wet cells) was dropped and adsorbed onto a porous cellulose nitrate membrane (20 mm diameter, 0.45 μm pore size; Advantec, Tokyo)

by suction. Another in the same membrane was placed on an immobilized microorganism membrane, and was re-adsorbed (thus making a sandwich) using the equipment above. The biofilm was placed on a chloride ion selective electrode and fixed in place using 200 mesh nylon and an 'O'-ring.

The microbial biosensor employed a chloride ion electrode to determine the presence of chloride ions released from TCE biodegradation by microorganisms. A chloride ion electrode (7024L, DKK, Tokyo) consisted of a working electrode with an ion-selective AgCl/Ag_2S membrane, and an Ag/AgCl reference electrode. The electrodes were connected to both an ion meter and an electronic recorder (EPR-200A, TOA electronics, Tokyo). The reaction cell was maintained at 30 °C using a water bath. When the potential reached equilibrium, TCE was added into the reaction cell, and was continuously monitored until the potential reached a constant value. The sensor response was evaluated as the difference between the potential before and after the addition of samples.

3. Results and discussion

3.1. Characterization of TCE-degrading microorganisms

We used the media described above to isolate microorganisms from soils and activated sludges. TCE-degrading microorganisms were successfully and repeatedly isolated from each sample that used toluene or tryptophan as carbon sources. After repeated streaking and isolation, a single pure colony for each carbon source was obtained. The taxonomic tests revealed that the cells were rod-shaped, motility, and gram negative. The isolated strains were identified to be *Pseudomonas* sp. strain ASA86 and *Burkholderia* sp. strain TAM17 for toluene and tryptophan, respectively. G + C contents were 62.3% and 65.7% for *Pseudomonas* sp. strain ASA86 and *Burkholderia* sp. strain TAM17, respectively.

Fig. 1 shows TCE biodegradation by TCE-degrading microorganisms. TCE biodegradation by *Pseudomonas* sp. strain ASA86 began 10 h after inoculation, and about 50% of the residue remained after 18 h (Fig. 1A). On the other hand, *Burkholderia* sp. strain TAM17 began degrading TCE 2 days after inoculation, and took 7 days to reach about 50% residual TCE (Fig. 1B). Strain TAM17 exhibited a very slow rate of TCE degradation compared to that of strain ASA86. Unlike some other aromatic solvents, aerobic degradation of TCE by *Burkholderia* sp. strain TAM17 required the induction of tryptophan. TCE biodegradation by tryptophan-induced cells has not been previously reported. Growth of TCE-degrading microorganisms for each carbon source was investigated. TCE-degrading microorganisms were incubated in 70-mL serum vials containing 20 mL of 0.2 g/L toluene- and tryptophan-containing media at 30 °C and 170 rpm. The lag periods were 11 and 24 h for strains ASA86, and TAM17, respectively. Strain ASA86 exhibited a very low growth rate, while TAM17 exhibited a high growth rate, the OD of which was six times greater than that at the stationary phase. Although the OD of strain ASA86 was low, its activity in the lag phase was more than 3-fold higher than that of strain TAM17. The results support the notion that strain ASA86 degraded TCE at a much faster rate than did strain TAM17 (Fig. 1).

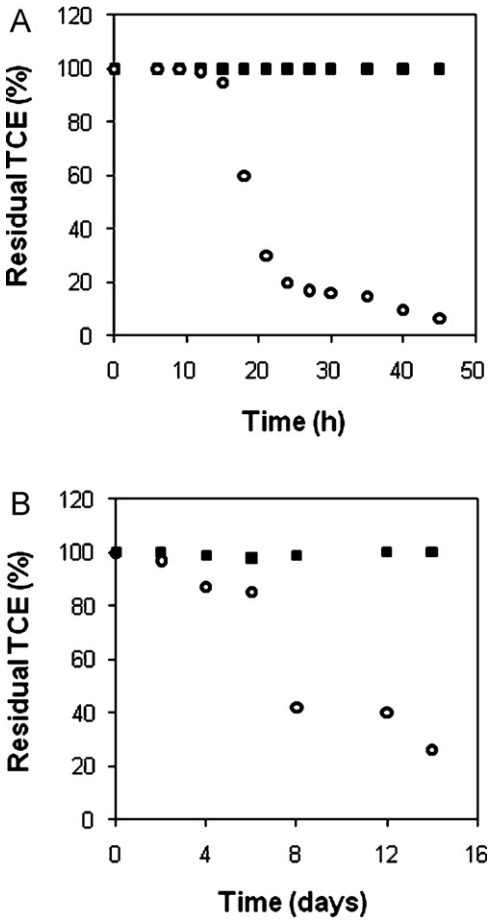

Fig. 1. Time courses of TCE biodegradation by TCE-degrading microorganisms. (A) *Pseudomonas* sp. strain ASA86. (B) *Burkholderia* sp. strain TAM17. The percentage of TCE biodegradation was calculated from the mean TCE concentration in three serum vials. (■) TCE degradation levels by uninoculated controls; (○) TCE degradation levels by either the strain ASA86 or TAM17.

3.2. Identification of toluene and TCE degradation genes

Aerobic degradation of TCE is mediated by a number of enzymes, including several enzymes that oxidize toluene [34]. Toluene is often required for the induction of TCE degradation by toluene oxygenases. Toluene degradation is carried out by an initial oxygenase, which forms toluene *cis*-dihydrodiol. This is followed by the dehydrogenation of a specific dehydrogenase to form 3-methylcatechol, which then decomposes to 2-hydroxy-6-oxohepta-2,4-dienoate by catecol-2,3-dioxygenase via the *meta*-ring cleavage pathway. The compound is further converted to CO_2 via the tricarboxylic acid cycle (Fig. 2A) [35]. In *P. putida* F1, the *tod* operon consists of 14 genes containing toluene dioxygenase (Fig. 2B) [36]. To identify toluene and the TCE degradation gene, *todC1* was amplified by PCR from chromosomal DNA with primers specific to the positions upstream and downstream of the gene. DNA fragments obtained from PCR amplification were 551 and 540 bp in size for strains ASA86 and TAM17, respectively (Fig. 2C). The DNA fragment of strain ASA86 was equivalent to the DNA size predicted from specific primers upstream and downstream of the *todC1* gene; however, this was not the case for strain TAM17. *Pseudomonas* sp. strain ASA86 had the same *todC1* sequence as *P. putida* F1 [32], whereas *Burkholderia* sp. strain TAM17 had *tecA1* sequence similar to that of *Burkholderia* sp. PS12 [37], with 85% identity at the nucleotide sequence level. Two genes were identified: one encoding for toluene dioxygenase of *todC1* and one for chlorobenzene dioxygenase of *tecA1*. On the other hand, in *B. cepacia* G4, TCE degradation is caused by toluene monooxygenase [38].

Aerobic degradation of toluene has been found to proceed via 6 different pathways, all of which are initiated by the oxidation of toluene by mono- or dioxygenase [36]. Toluene is continuously converted to toluene *cis*-dihydrodiol by *P. putida* F, benzyl alcohol by *P. putida* PaW1, *o*-cresol by *B. cepacia* G4, *m*-cresol by *Ralstonia pickettii* PKO1, *p*-cresol by *P. mendocina* KR1, and *Burkholderia* sp. JS150. This suggests that the degradation of toluene and TCE by

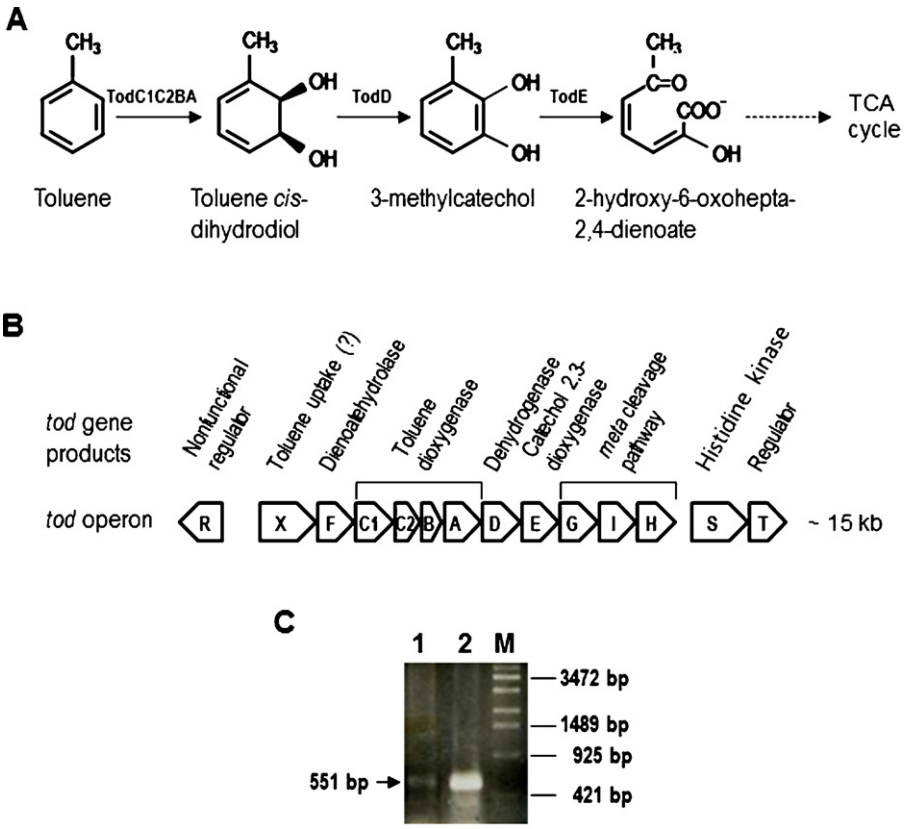

Fig. 2. (A) Catabolic pathway of toluene by toluene dioxygenase of TCE-degrading microorganism. Gene products catalyzing each step are indicated in panel B. (B) Genetic organization of the *tod* operon degrading toluene in *P. putida* F1. Gene products are described over each gene. (C) Electrophoretic analyses of PCR products of the *todC1* gene in the chromosomes of TCE-degrading microorganisms. Lane 1, *Burkholderia* sp. strain TAM17; lane 2, *Pseudomonas* sp. strain ASA86; lane M, λ DNA marker digested with *Sty*I. Samples were analyzed on a 1% agarose gel.

strain ASA86 may be similar to the cometabolic pathway of *P. putida* F1, because the strain ASA86 has the toluene dioxygenase enzyme.

3.3. TCE biodegradation by toluene-induced cells

To investigate the tendencies of TCE and toluene degradation, *Pseudomonas* sp. strain ASA86 was cultured in a 70-mL serum vial containing 35 mL medium, 1 mg/L TCE, and 200 mg/L toluene at 30 °C and 170 rpm. Biodegradation analyses of TCE and toluene were carried out by headspace gas chromatography. Biodegradation relationships between TCE and toluene by strain ASA86 are shown in Fig. 3. TCE degradation did not occur in the absence of toluene (data not shown). Toluene degradation began after 10 h of incubation, and TCE degradation after 12 h. After 18 h, the residual amounts were 86.1% and 97% for toluene and TCE, respectively. The degradation of toluene tended to be similar to the growth pattern of strain ASA86. Toluene was entirely degraded by 21 h, but 83.4% of TCE was degraded in the same time, and was almost completely degraded by 50 h. The results indicated that TCE degradation by strain ASA86 was induced by toluene.

Chloride ions in solution, released during TCE biodegradation, were also determined by a DR2000 spectrophotometer using mercuric thiocyanate. Concentration of chloride ions in solution increased with TCE biodegradation (Fig. 3). However, although TCE degradation was 83.4% at 21 h, only 13% chloride ions were released. This result suggests that biodegraded TCE may form intermediates, such as hydroxy- or oxochlorinated compounds, before converting to CO_2. The mechanisms of the TCE biodegradation are well unknown. Oxidation of TCE by oxygenases could be carried out from an initial attack by either a mono- or dioxygenase, which form an epoxide or dioxetane intermediate, respectively [10,35]. These could be converted to hydroxy- or oxochlorinated compounds, which then decompose to CO_2 through dichloroacetic acid, glyoxylic acid, formate, and carbon monoxide. Generally, increase in chloride ion release was found to be proportional to TCE degradation.

3.4. Evaluation of the biosensor for TCE determination

Strain ASA86 was employed as the bioelement to evaluate the biosensor for TCE determination. The biosensor system consisted of a chloride ion electrode with the biofilm, and an electronic

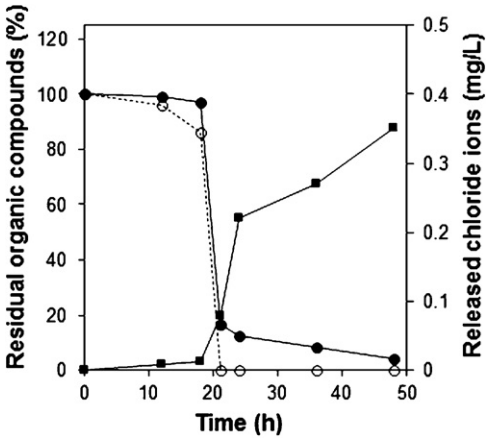

Fig. 3. Biodegradation of organic compounds and release of chloride ion by *Pseudomonas* sp. strain ASA86. (●) TCE; (○) toluene; (■) chloride ion.

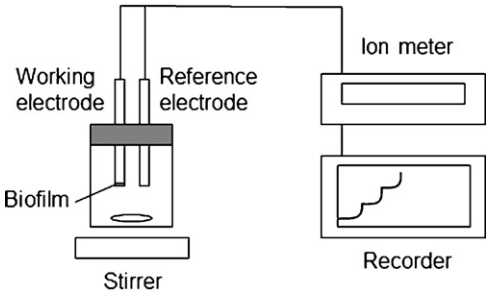

Fig. 4. Schematic representation of the TCE biosensor of the batch type using *Pseudomonas* sp. strain ASA86.

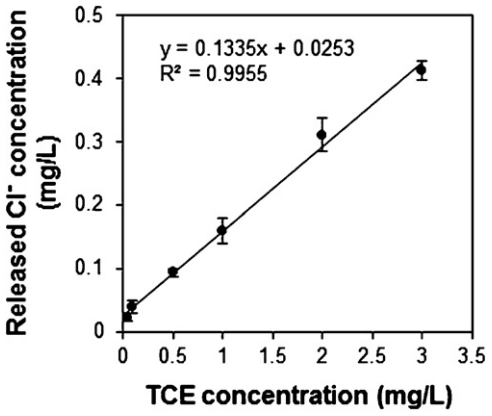

Fig. 5. The correlation between TCE and free chloride ion by TCE biosensor. Each point was a mean of 3 experiments, and error bars represent standard deviation.

recorder (Fig. 4). The biosensor was inserted into the reaction cell with 50 mL of 10 mM phosphate buffer. The reaction cell had very little headspace to suppress the volatilization of TCE, because the vapor pressure of TCE was approximately 65 mm Hg at 30 °C [27,28]. Chloride ions, released during the biodegradation of TCE, were confirmed by a chloride ion electrode with the biofilm. Analytical characteristics of the TCE biosensor are shown in Fig. 5. Calibration was performed using the response data at the steady state. A linear relationship was observed below 3 ppm TCE. The biosensor response could be detected to 0.05 ppm TCE. The relative standard deviation was 6.1 at 0.5 ppm TCE. The response is similar to that of microbial biosensor using *Pseudomonas aeruginosa* JI104 [39], and extremely higher than 0.2 ppm of *Pseudomonas fluorescens* A506 biosensor [28]. In Japanese environmental quality standard for industrial water and waste water, limit value on emission of the pollutant is <0.3 ppm. The response time of the biosensor was less than 10 min. To investigate the reproductivity and stability, the biosensor was stored in phosphate buffer and periodically exposed to 0.5 ppm TCE. The biosensor showed stable responses during a 2 day period. The results show that strain ASA86 is a suitable bioelement for the TCE biosensor. Furthermore, the sensitivity of the microbial sensor describes its ability to monitor TCE in ground water, and drinking water. The TCE biosensor can be functionally equivalent to the conventional TCE determination methods, which are required pretreatment of samples including solvent extraction and concentration.

4. Conclusion

To develop a biosensor for TCE determination, TCE-degrading microorganisms were isolated. The microorganisms isolated were aerobic bacteria; *Pseudomonas* sp. strain ASA86 and *Burkholderia* sp. strain TAM17, which used toluene and tryptophan, respectively, as carbon sources. Thus far, tryptophan has been not reported as a carbon source for the induction of TCE biodegradation. Two bacterial strains had oxygenases for TCE degradation: the genes were *todC1* and *tecA1* for strains ASA86 and TAM17, respectively. Further, a novel microbial TCE biosensor was developed using the strain ASA86. The detection limit of the biosensor was 0.05 ppm TCE, which was below limit value for the pollutant emission from industrial water and waste water. Then, the TCE biosensor demonstrates sufficient performance for the TCE determination of industrial effluents. Interference on the response of the biosensor will be investigated. The TCE biosensor, unlike the conventional methods, is applicable to in situ determination and on-line monitoring.

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