WALAILAK JOURNAL
Applied Sciences
http://wjst.wu.ac.th https://doi.org/10.48048/wjst.2021.7306

Reductive Dechlorination of 1,2-dichloroethane to Ethylene by Anaerobic Enrichment Culture Containing *Vulcanibacillus* spp.

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Received: 10 August 2019, Revised: 26 March 2020, Accepted: 27 April 2020

Abstract

Bioremediation has been widely used for clean-up of 1,2-dichloroethane (DCA) at contaminated sites. Only a small number of specific anaerobes, halorespiring bacteria (HRB), have been reported to degrade DCA. The goals of this research were the screening and isolation of HRB with capable dechlorination of DCA. HRB were screened and isolated from 7 enrichment cultures (ES1-ES7), from DCA-contaminated soils, on bicarbonate-buffered basal salts medium containing 10 mM acetate and 250 µM DCA under anaerobic conditions and analyzed by gas chromatography. The results showed that the mixed cultures of ES3 and ES5 could reductively dechlorinate DCA to ethylene via a direct reductive dihaloelimination pathway. In particular, ES5 showed rapid transformation of 250 µM DCA to ethylene within 6 days at 30 °C. Specific microbial populations of *Vulcanibacillus* spp., elucidated by polymerase chain reaction-denaturing gradient gel electrophoresis, were found only in ES3 and ES5, which was related to reductive dihaloelimination activities on DCA. A 16S rRNA gene analysis of isolate es5d8 from mixed culture ES5 revealed 2 strains of *Vulcanibacillus* spp. (KKU-DCA1 and KKU-DCA2) concerned with dechlorinating DCA. These findings suggested that *Vulcanibacillus* spp. were HRB that have the potential for detoxifying DCA.

Keywords: Bioremediation, 1,2-Dichloroethane, Halorespiring bacteria, Reductive dihaloelimination, *Vulcanibacillus*

Introduction

1,2-dichloroethane (DCA) is a chlorinated hydrocarbon and is widely used as a solvent and precursor in the manufacture of vinyl chloride monomer (VCM) for synthetic textile fibers, rubbers, plastics, and other materials [1]. The utilization of DCA is increasing in many countries around the world. In recent years, the worldwide DCA production reached 39.4 million tons [2], and it is expected to reach 58 million tons by 2027 [3]. A large amount of DCA is released into the soil and water and is known to cause cancer in humans [4]. The causes of contamination of the environment have been both accidental spills and poor handling. Due to DCA being denser than water, when it is released into soil, it permeates the groundwater, where it tends to stay for many years [5]. Decontaminating such sites is not easy. Abiotic degradation of DCA may occur; however, this reaction is very slow. Modelling groundwater conditions, the half-life of DCA present in 1 mM total sulfide pH 7 at 5 °C is 23 years [6]. Therefore, development of new remediation technologies for contaminated sites is necessary.

In recent years, various technologies, including physical, chemical, and biological methods, have been developed and applied to solve environmental pollution [7,8].

Among these technologies, bioremediation is the most suitable for decontaminating chlorinated compound-polluted soil and groundwater, because it is relatively inexpensive and environmentally friendly [9]. Bioaugmentation and biostimulation processes are frequently used for contaminated sites in many countries [10,11]. Among microorganisms, there are both aerobic and anaerobic bacteria that can completely degrade DCA. However, soil and groundwater often have limited oxygen and energy sources for microorganisms. Therefore, anaerobic bacteria are required for the field application of remediation technologies.

Several bacterial strains, defined as halorespiring bacteria (HRB), use chlorinated compounds for growth as electron acceptors in reductive dehalogenation reactions under anaerobic conditions [12]. Reports on chlorinated solvent bioremediation have found that 3 phyla, *Proteobacteria* (classes: *Deltaproteobacteria*, and *Epsilonproteobacteria*) [13,14], *Firmicutes* (class: *Clostridia*) [15], and *Chloroflexi* (class: *Dehalococcoidia*) [16] can dechlorinate chlorinated hydrocarbons. However, there are some species of the genera *Methanobacterium*, *Desulfitobacterium,* and *Dehalococcoides* that are capable of complete metabolic reduction of DCA and vinyl chloride (VC) to ethylene [17-19]. *Methanobacterium thermoautotrophicum* can dechlorinate DCA to ethylene cometabolically at 63 °C [17], while for *Desulfitobacterium dichloroeliminans* DCA1 to be an anaerobic dehalorespiring bacterium, it requires menaquinone as an essential cofactor for growth in pure culture [18]. Some pure cultures of the genus *Dehalococcoides*, such as *D. mccartyi* strain 195, can reductively dechlorinate DCA to 94 % ethylene and 1 % VC [19]. Even though this previous research found that *Dehalococcoides* strains reductively dechlorinate DCA with high efficiency, this genus usually grows slowly, with a doubling time of 1-2 days [20]. Moreover, reductive dechlorination of volatile organic compounds by *D. mccartyi* strain 195 has low efficiency and is incomplete when the strain is maintained in a pure culture [19]. The incomplete microbial reductive dechlorination of DCA leads to accumulation of the known carcinogen VC, which is more toxic than the parent compound [21].

For efficient clean-up of severely contaminated sites, it is necessary to have many kinds of effective microorganisms. Therefore, in this study, HRB were enriched with DCA-contaminated soil collected in Rayong Province, Thailand. The anaerobic enrichment cultures were screened and characterized with regard to the detoxification of DCA. HRB were identified using gas chromatography (GC) and polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE).

Materials and methods

Chemicals

Analytical grade reagents: DCA (99.5 % purity, Sigma-Aldrich); VC (99.5 % purity, Sigma-Aldrich), and ethylene (Kasidith Trading, Thailand) were used as standards. A gas mixture containing 5 % hydrogen (H_2), 5 % carbon dioxide (CO_2), and 90 % nitrogen (N_2; Kasidith Trading, Thailand) was used as an anaerobic purge gas in anaerobic chambers. Acetate (sodium salt, 99 %, analytical grade, Ajax Finechem, Australia) was employed as a carbon source.

Collection of soil samples

Microorganism isolated in this study were obtained from soil samples contaminated with DCA. Soil samples were collected from 7 DCA contamination sites on the Map Ta Phut Industrial Estate (MTPIE), Rayong Province, Thailand (coordinates: 12°43′33″N 101°10′20″E). The soil samples were collected at a depth between 1 and 5 m using the Geoprobe drilling technique. Samples were packed in sealed dark plastic bags, kept on ice, and transported to the laboratory for study of enrichment cultures.

Enrichment and screening of halorespiring bacteria

The seven DCA-contaminated soil samples were used to enrich cultures of HRB and were assigned the names ES1-ES7. Using a Bactron anaerobic chamber (Shelden Manufacturing, USA), 2 g of soil samples were transferred into 60 ml bottles containing 20 ml bicarbonate-buffered basal salts medium (BS medium) [22,23]. BS medium contained 10 mM acetate, 30 mM $NaHCO_3$, 0.5 mM DL-dithiothreitol, and 0.2 mM L-cysteine. Resazurin sodium salt was supplied to the medium at 0.001 g/l to act as an

indicator of reductive conditions. For sterilization, the medium was subjected to autoclaving at 121 °C, 15 psi for 40 min, and was then transferred to an anaerobic chamber. The pH of the medium was adjusted to 7 using 1 N NaOH or 1 M HCl. DCA was added to the culture medium at a final concentration of 250 µM. All culture bottles were capped with Teflon and aluminum caps and incubated at 30 °C in the dark for 14 days. Following this, the initial culture was sub-cultivated 3 times (every 7 days) by diluting 10 % v/v into fresh medium, and their optical densities (OD) at 600 nm were determined by Spectronic 200 spectrophotometer (Thermo Fisher Scientific, USA).

The mixed cultures ES1-ES7 were screened for reductive dechlorination of DCA in 20 ml BS medium containing 10 mM acetate and 250 µM DCA in 60 ml bottles incubated under anaerobic conditions at 30 °C for 14 days. Abiotic controls were set up with an autoclaved mixed culture (control 1) and uninoculated BS medium (control 2) that were otherwise prepared as per the enrichment culture bottles. After 7 and 14 days of incubation, all of the cultures were determined by using GC to measure the concentration of DCA and its product.

Time course of reductive dechlorination

The time courses of reductive dechlorination of DCA by cultures were observed in triplicate serum bottles (20 ml liquid, 40 ml headspace). The inoculum cultures were done in BS medium, which was the same as the enrichment culture bottles. After 7 days, 10 % of the highly enriched cultures with complete conversion of DCA to ethylene was transferred to 20 ml BS medium containing 10 mM acetate and 250 µM DCA. All culture media were incubated at 30 °C for 7 days. Every day, the concentrations of DCA and its products were determined by GC.

Analytical methods for determining DCA and ethylene

The concentrations of DCA and ethylene were determined by the headspace technique [24] using a GC-14B GC (Shimadzu, Kyoto, Japan) with flame ionization detector and capillary column DB-624, length 30 meter, I.D. 0.53 millimeter (J&W Scientific, USA). The injector, detector, and column temperature were set at 200, 250, and 60 °C, respectively. Helium was the carrier gas with a flow rate of 5 ml/min.

Microbial community analysis

Total DNA of each culture was extracted using the ZR soil microbe DNA MiniPrep^{TM} (Zymo Research, USA), following the manufacturer's instructions. The extracted DNA was stored at –20 °C and used as a template for amplifying the 16S rRNA gene. DCA-reducing bacterial communities in the mixed cultures were determined by nested PCR-DGGE analyses, using the V3 region on the 16S rRNA gene [25]. Firstly, the primer pairs 8F and 1512R were used to amplify a fragment of the 16S rRNA gene (1,500 bp) [26]; then, the primer pairs 338F and 518R were used to amplify a fragment of the V3 region (200 bp). The 5' end of primer 338F was attached to a GC-clamp of about 40 bp to make a melting domain for gel electrophoresis [25].

DGGE analysis of the variable V3 region fragments was performed with a TV400-DGGE system (SciePlus). PCR products (30 µl) were loaded and separated in 20.5×20.0 cm^2 gels containing 8 % (w/v) polyacrylamide (37:5:1; Amresco, USA), with a 35 - 60 % linear denaturant gradient (prepared from 100 % denaturant gels containing 7 M urea and 40 % v/v formamide). Electrophoresis in 0.5 TAE buffer was at a constant voltage of 20 V at 60 °C for 10 min, and then at 70 V for 16 h. Gels were stained and washed in 0.5 mg/l ethidium bromide solution (10 min) and distilled water (20 min), respectively. The DGGE profiles were photographed and analyzed with UV transillumination and gel documentation (Synoptic Ltd, UK).

The dominant bands were selected and re-amplified using primer 338F without a GC clamp and 518R, then purified using a GF-1 AmbiClean Kit (Vivantis, Malaysia). The nucleotide sequences were obtained using the BigDye Terminator v3.1 cycle sequencing kit (First BASE Laboratories, Malaysia) and compared with those in GenBank using the BLASTN function (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Isolation and identification of halorespiring bacteria

HRB were isolated from mixed cultures exhibiting DCA-dechlorinating activity by the roll tube technique [27]. To determine the ability of pure cultures in reductive dechlorination of DCA, the amounts of ethylene from the culture samples were measured by GC. In addition, partial 16S rRNA gene fragments were amplified from the pure cultures and confirmed by PCR-DGGE.

The 16S rRNA genes from the mixed cultures and roll tube were amplified and cloned with the pGEM®-T Easy Vectors cloning kit (Promega, USA), following the manufacturer's protocol. Full-length sequence inserts in the plasmid were selected and sequenced using the BigDye Terminator v3.1 cycle sequencing kit (First BASE Laboratories, Malaysia). The partial sequence was analyzed by using the BLAST and ClustalW program [28]. Phylogenetic trees were constructed using the neighbor-joining method (1,000 bootstrap replicates) with MEGA7 [29,30]. Finally, the nucleotide sequences from this study were submitted to GenBank under accession number MG761727.1 and MG761728.1 for *Vulcanibacillus* enrichment culture clones KKU-DCA1 and KKU-DCA2, respectively.

Statistical analysis

The experiments were conducted in triplicate, with results being presented as mean ± standard deviation. Duncan's one-way ANOVA multiple comparison was used to analyze the differences among the group of samples for more than 3 groups of samples. The statistical analysis was performed at 95 % confidence intervals ($\alpha = 0.05$) by SPSS version 17.0.

Results and discussion

Enrichment and screening of halorespiring bacteria

HRB mixed cultures, namely ES1-ES7, were enriched from DCA-contaminated soil samples with BS medium containing 10 mM acetate and 250 µM DCA, under anaerobic conditions at 30 °C. Essential factors for reductive dechlorination activity of HRB are acetate (carbon source), H_2 (electron donor), and DCA [16]. The reductive dechlorination of 250 µM DCA by HRB mixed cultures was determined by GC, as shown in **Table 1**. The results showed that only mixed cultures of ES3 and ES5 could reductively dechlorinate DCA to ethylene at 7 days of incubation, as 85.9 and 100.0 %, respectively. Meanwhile, reductive dechlorination activity of ES3 completely dechlorinated DCA to ethylene within 14 days. The mixed culture ES5 showed the highest detoxifying activity, in that the concentration of ethylene increased (100.0 %) as the DCA concentration decreased, after 7 and 14 days of incubation. Results of other mixed cultures ES1, ES2, ES4, ES6, ES7, and controls were not found to have reductive dechlorination activity at 7 and 14 days of incubation.

There were 2 mixed cultures, ES3 and ES5, that could produce ethylene as an end product. From previous reports, ethylene can be the product of microbial transformation under anaerobic conditions [12]. The group of microorganisms that carries out biotransformation in anaerobic conditions is halorespiring bacteria, including the genera *Methanobacterium*, *Desulfitobacterium*, and *Dehalococcoides* [17-19]. These bacteria use DCA for growth as an electron acceptor and produce ethylene as the final product. Therefore, it is possible that mixed cultures ES3 and ES5 included halorespiring bacteria, exhibiting DCA dechlorination to ethylene.

Table 1 Reduction of DCA and increase of ethylene by mixed cultures (ES1-ES7) incubated for 7 and 14 days.

Sample name	Day 7		Day 14	
	DCA (% reduction)	Ethylene (% increasing)	DCA (% reduction)	Ethylene (% increasing)
ES1	4.8 ± 1.5	0.0 ± 0.0	5.6 ± 3.7	0.0 ± 0.0
ES2	3.2 ± 1.2	0.0 ± 0.0	8.6 ± 1.4	0.0 ± 0.0
ES3	85.9 ± 5.3	83.8 ± 0.2	100.0 ± 0.0	100.0 ± 0.0
ES4	10.8 ± 8.1	0.0 ± 0.0	19.8 ± 5.1	0.0 ± 0.0
ES5	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
ES6	13.9 ± 1.3	0.0 ± 0.0	17.7 ± 0.5	0.0 ± 0.0
ES7	7.7 ± 2.0	0.0 ± 0.0	18.8 ± 5.5	0.0 ± 0.0
Control1	3.8 ± 1.5	0.0 ± 0.0	5.6 ± 1.4	0.0 ± 0.0
Control2	3.4 ± 2.1	0.0 ± 0.0	9.8 ± 1.1	0.0 ± 0.0

Note: ± = Standard deviations for 3 replicate cultures.

Analysis of bacterial communities in enrichment cultures by PCR-DGGE

Ethylene was detected in the ES3 and ES5 mixed cultures, suggesting that they contained halorespiring bacteria that exhibited DCA-dechlorinating activity. Therefore, the main bacterial populations of ES3 and ES5 were compared to those of the other mixed cultures, which could not dechlorinate DCA. The dominant bacteria of mixed cultures ES1-ES7 were determined by using the PCR-DGGE technique, as shown in **Figure 1**. The ten dominant bands from each mixed culture were sequenced and then identified using Blast on GenBank (**Table 2**).

As shown in **Figure 2**, DGGE profiles exhibiting DCA-dechlorinating mixed cultures of ES3 and ES5 revealed that DNA bands 5 and 6 differed from the other mixed cultures. The sequences of DNA bands 5 and 6 were 99 % similar to the 16S rRNA gene of *Vulcanibacillus* sp. b-5, while other bands, such as DNA band 1, were found in DGGE profiles of mixed cultures ES1 and ES7, and they were 100 % similar to *Pseudomonas aeruginosa*. DNA band 2 appeared in mixed cultures ES1 and ES2 and showed 100 % similarity to *Acinetobacter ursingii* strain blaTEM-116. DNA band 3 was found in mixed culture ES5 and showed 99 % similarity to *Bacillus korlensis* strain RPCal-HMN_10-2-5. DNA bands 4, 7, 9, and 10 were present in DGGE profiles of mixed cultures of ES4 and ES6, and they were 98 % similar to *Paenibacillus xylanisolvens*, *P. validus* strain 1VC IRAN, uncultured *Paenibacillus* sp., and bacterium EA10-ST-8, respectively. DNA band 8 appeared in ES2, ES3, and ES5 and revealed a 100 % match to the sequence of *Desulfotomaculum aeronauticum*. These bacteria were phyla *Firmicutes* and *Proteobacteria.*

By comparison, mixed cultures ES3 and ES5, exhibiting DCA-dechlorinating activity, contained *Vulcanibacillus* sp. (bands 5 and 6) and *Desulfotomaculum aeronauticum* (band 8). In a previous study, *Vulcanibacillus* sp., strictly anaerobic, was classified as a nitrate-reducing bacterium [27], while *D. aeronauticum,* a thiosulfate-reducing bacterium, was often found in soil [31]. Some species of the genus *Desulfotomaculum*, such as *D. aquiferis* and *D. profundi*, were isolated from a deep natural gas storage aquifer [32]. Notably, *D. aeronauticum* was also present in ES2; however, ES2 did not reductively dechlorinate DCA. Therefore, it is possible that this species may not direct reductive dechlorination of DCA. The dominant band *Vulcanibacillus* sp. was only found in DGGE profiles of ES3 and ES5. These findings suggest that *Vulcanibacillus* in ES3 and ES5 correlate with reductive dechlorination of DCA to ethylene under anaerobic conditions.

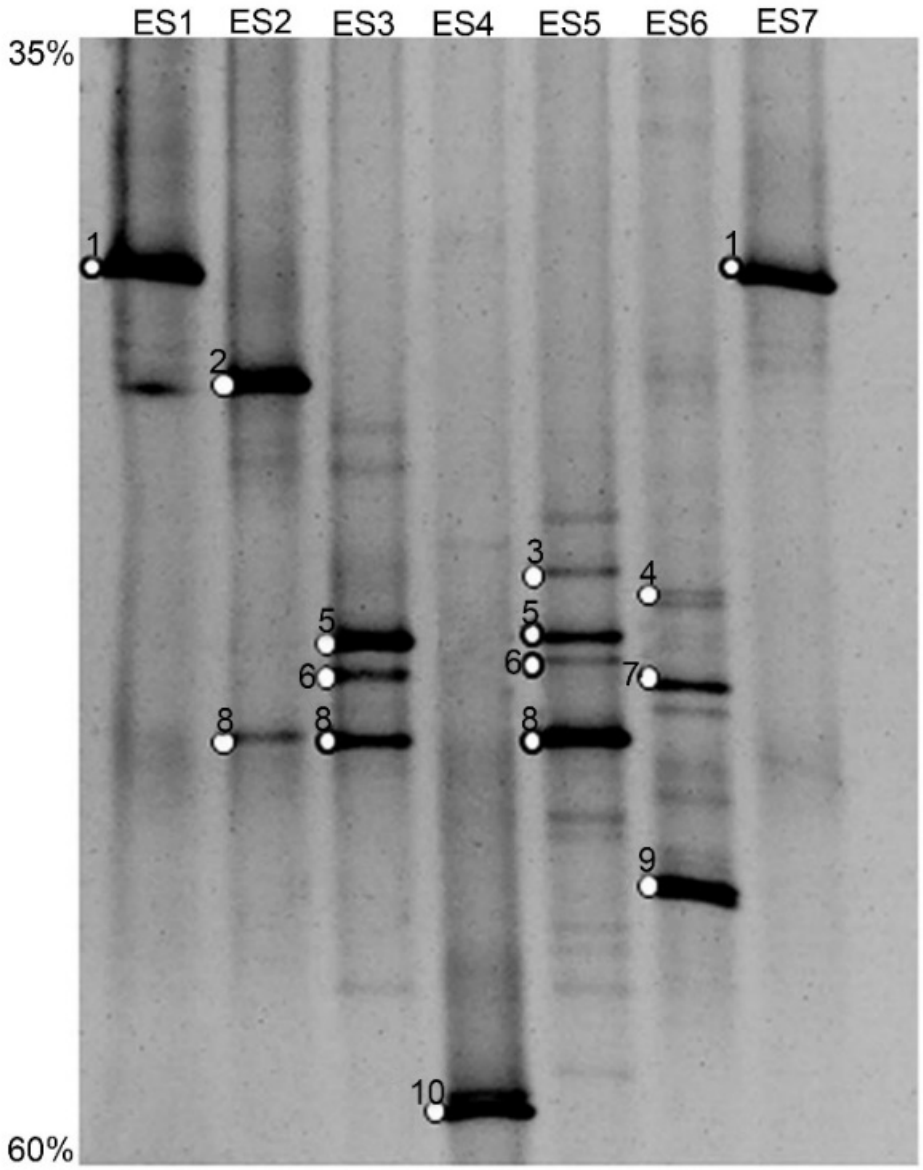

Figure 1 DGGE profiles of V3 region of 16S rRNA gene from mixed cultures. ES1-ES7: mixed cultures growing on BS medium containing acetate and DCA incubated under anaerobic conditions at 30 °C for 14 days.

Table 2 Blast result of V3 region on 16S rRNA gene of mixed cultures (ES1-ES7).

Band	Species or Genus with Highest Sequence Similarity	Sequence Similarity (%)	Accession Number	Phylum
1	*Pseudomonas aeruginosa*	100	FM957532.1	*Proteobacteria*
2	*Acinetobacter ursingii* strain blaTEM-116	100	MH113156.1	*Proteobacteria*
3	*Bacillus korlensis* strain RPCal-HMN_10-2-5	99	KX417393.1	*Firmicutes*
4	*Paenibacillus xylanisolvens*	98	LT853723.1	*Firmicutes*
5	*Vulcanibacillus* sp. b-5	99	KF933847.1	*Firmicutes*
6	*Vulcanibacillus* sp. b-5	99	KF933847.1	*Firmicutes*
7	*Paenibacillus validus* strain 1VC IRAN	98	MH159206.1	*Firmicutes*
8	*Desulfotomaculum aeronauticum*	100	EU882404.1	*Firmicutes*
9	Uncultured *Paenibacillus* sp.	98	HE681432.1	*Firmicutes*
10	Bacterium EA10-ST-8	98	JF418060.1	*Firmicutes*

Nucleotide sequence bands were obtained from DGGE gel of 7 mixed cultures samples which were grown in BS medium containing acetate and DCA incubated under anaerobic conditions at 30 °C for 14 days.

Time course of reductive dechlorination of DCA by ES3 and ES5

The mixed cultures ES3 and ES5 could reductively dechlorinate DCA to ethylene under 7 days of incubation, as shown in **Figure 2**. The reductive dechlorination activity of DCA by mixed culture ES3 started after 2 days of incubation (**Figure 2A**). The maximum dehalogenation rate of DCA to ethylene was observed at day 2 to day 3, with an average dechlorination rate of 38 µM/day. However, mixed culture ES3 had no potential for complete detoxification of 250 µM DCA to ethylene within 7 days of incubation. Meanwhile, the results from mixed culture ES5 showed even more dramatic dechlorination of 250 µM DCA to ethylene after 2 days of incubation, and completely dechlorinated within 6 days of incubation (**Figure 2B**). On days 2 and 4 of incubation, ES5 showed the highest dechlorination rates, at approximately 91 and 99 µM/day, respectively. Considering the dichlorination behavior of the mixed cultures ES3 and ES5, the conversion of DCA to ethylene was observed after 2 days of incubation. This suggests that the bacteria need a period of time for adaptation in DCA-contaminated conditions. This result is similar to that of Maymo-Gatee *et al*. [33], who reported that DCA was converted mainly to VC and ethylene after 2 days of incubation by *Dehalococcoides mccartyi* strain 195. Nevertheless, some bacteria strain need a long period for the degradation process. The reductive dechlorination of DCA (420 µM) by *D. mccartyi* strain BTFO8 to VC and ethylene was much slower, with 55 days of incubation [19].

In this study, ethylene was the primary product and VC, as an intermediate, was not found. This demonstrated that mixed cultures ES3 and ES5 dechlorinated DCA to ethylene via a direct dichloroelimination mechanism under anaerobic conditions. The mechanism of reductive dichloroelimination involves 2 chlorine atoms being removed from the same alkane molecule and replaced with hydrogen atoms, leading to the formation of ethylene [19]. In the last 10 years, *Desulfitobacterium dichloroeliminans* strain DCA1, *Dehalogenimonas lykanthroporepellens* strain BL-DC-9(T), *Dehalococcoides mccartyi* strain 195, and *D. mccartyi* strain BTF08 have been shown to convert DCA to VC and ethylene [18,19,34]. However, this result is different from the reductive dechlorination activity of *Dehalococcoides mccartyi* strain 195 and *D. mccartyi* strain BTF08 that dechlorinated DCA to ethylene and VC [19]. It is known that VC is more toxic than the parent compound [21].

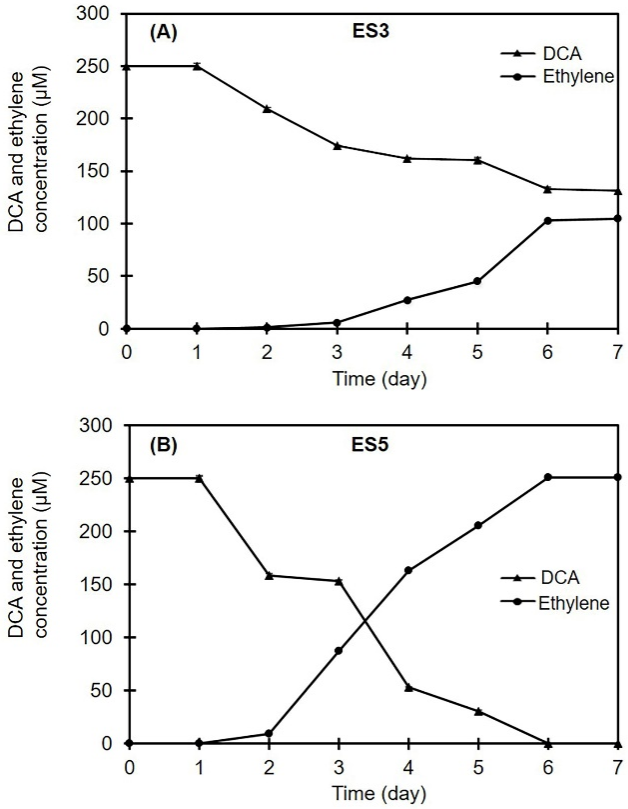

Figure 2 Time courses of reductive dechlorination of DCA activity by (A) ES3 and (B) ES5. Error bars are standard deviations for 3 replicate cultures.

Isolation and identification of DCA-dechlorinating bacteria

The isolates es5d3 and es5d8 were isolated from ES5 by roll tube, and they exhibited DCA dechlorination to ethylene. The V3 band of 16S rRNA gene of es5d3 and es5d8 were confirmed as *Vulcanibacillus* sp. by PCR-DGGE (**Figure 3**). The results show that the same single band from es5d3 and es5d8 was found, and it matched with DNA band 5 from ES5. The DNA band 5 from ES5 was identified as *Vulcanibacillus* sp. It suggested that *Vulcanibacillus* is a halorespiring bacterium that exhibits DCA-dechlorinating activity.

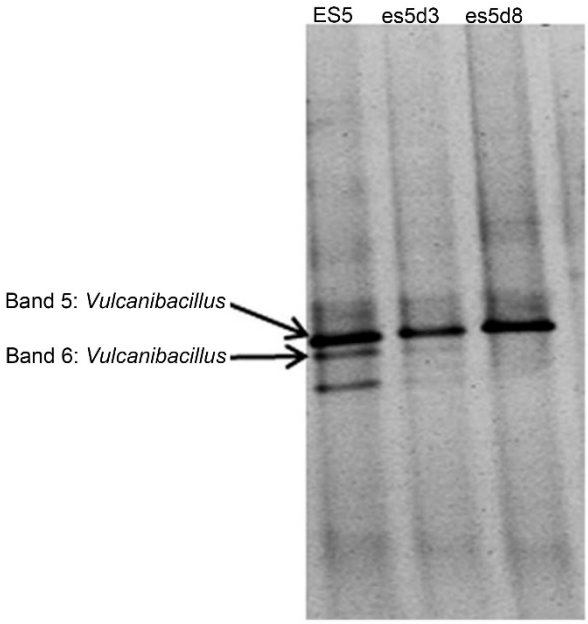

Figure 3 DGGE profiles of V3 region of 16S rRNA gene from mixed ES5 and isolated es5d3 and es5d8 cultures.

To specifically obtain a full-length 16S rRNA gene of *Vulcanibacillus* in culture es5d8, its 16S rRNA gene was amplified, cloned, and analyzed with the DGGE technique, as shown in **Figure 4**. DGGE analysis of the V3 region band of *Vulcanibacillus* was detected in 7 clones (C1-C7). The results show that the V3 bands of C1 and C2-C7 were the same as *Vulcanibacillus* band 6 and band 5 of enrichment culture ES5, respectively. This finding indicates that 2 species of *Vulcanibacillus* from isolated es5d8 are concerned with reductive dechlorination of DCA to ethylene under anaerobic conditions.

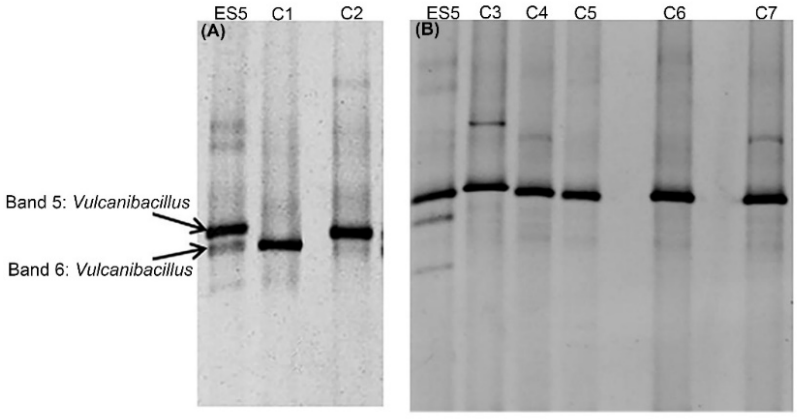

Figure 4 DGGE profiles of V3 region of 16S rRNA gene from mixed ES5 and clones of es5d8. (A) Clone nos. C1-C2; (B) clone nos. C3-C7; Lane ES5, mixed culture of ES5 as positive control.

Two full-length 16S rRNA genes of *Vulcanibacillus* inserted in the plasmids of clones C1 and C2 were 1,500 bp and 1,591 bp, respectively. BLAST results from the GenBank database showed that 16S rRNA gene sequences from clones C1 and C2 had 97 and 98 % similarities to the 16S rRNA genes of *Vulcanibacillus* sp. b-5 (KF933847.1) and uncultured *Vulcanibacillus* sp. (KC594801.1), respectively. The 16S rRNA sequence alignment by BLAST showed that the gene sequence from clone C1 had 2 % difference from clone C2, with query cover 97 %. They were assigned names and submitted to the National Center for Biotechnology Information as *Vulcanibacillus* enrichment culture clones KKU-DCA1 (clone C2) and KKU-DCA2 (clone C1). Phylogenetic trees presented were related with the *Bacillaceae* family and the closely related *Vulcanibacillus* b-5^T branch, as shown in **Figure 5**. The accession number for *Vulcanibacillus* enrichment culture clones KKU-DCA1 and KKU-DCA2 were MG761727.1 and MG761728.1, respectively. *Vulcanibacillus* sp. was classified as phylum *Firmicutes*, class *Bacilli*, order *Bacillales*, and family *Bacillaceae*. In another study, *Vulcanibacillus* sp. was enriched from deep-sea hydrothermal core samples at a temperature range of 37-60°C and was reported as nitrate-reducing bacteria [27]. Therefore, this study provided new insights into *Vulcanibacillus* spp. enrichment culture clones KKU-DCA1 and KKU-DCA2 as HRB and as DCA-dechlorinating bacteria. It is a significant advance for bioremediation of DCA-contaminated sites because of the complete degradation of, and no harm from, VC.

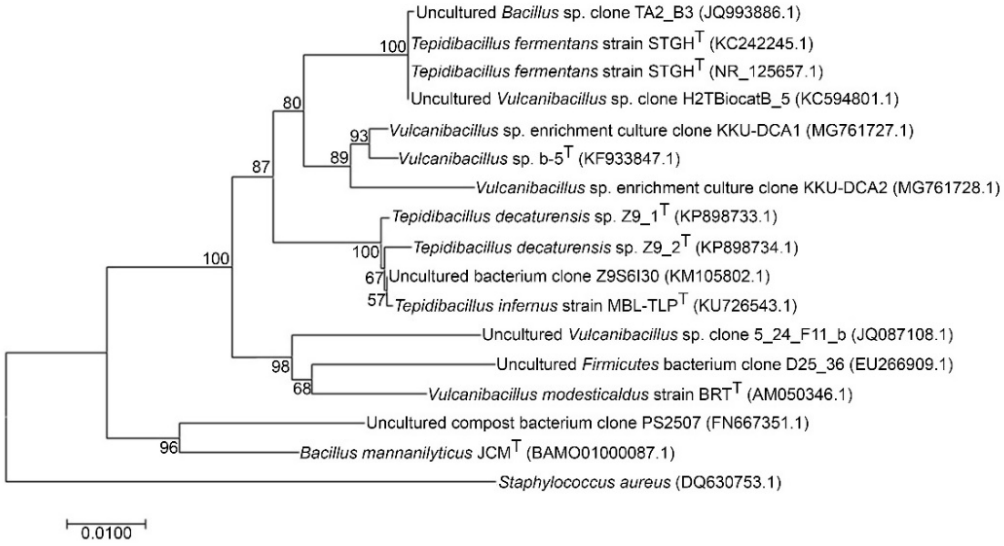

Figure 5 Phylogenetic tree based on 16S rRNA gene sequences of KKU-DCA1 and KKU-DCA2 clones from isolated es5d8 and closely-related taxa of the *Bacillaceae* family. The sequence of *Staphylococcus aureus* was used as the out group.

Conclusions

This study discovered the anaerobic enrichment culture containing *Vulcanibacillus* spp. that could reductively dechlorinate DCA to ethylene via the reductive dihaloelimination pathway. The complete dechlorination of DCA to ethylene was obtained within 7 - 14 days of incubation at 30 °C. Microbial diversity results from mixed cultures showed that *Vulcanibacillus* spp. was a major reducing bacteria, which was related with reductive dechlorination activity of DCA. The 16S rRNA gene analysis of isolate es5d8 from ES5 revealed 2 strains of *Vulcanibacillus* spp. enrichment culture clones KKU-DCA1 and KKU-DCA2. This finding indicated that *Vulcanibacillus* spp. was a DCA-dechlorinating or

halorespiring bacteria. They may be a possible remediation option for DCA removed by bioaugmentation technique through added dechlorinating bacteria and essential nutrients, such as acetate and H_2, that could metabolize target pollutants in contaminated sites.

Acknowledgements

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Research Center for Environmental and Hazardous Substance Management, Khon Kaen University. The authors would like to thank the Department of Pollution Control, Thailand, for collecting soil samples and for analysis. In addition, the authors would like to thank the Department of Biochemistry and the Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Thailand, for access to their laboratory equipment.

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