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Contributed Paper

Detection of Bacterial Communities in Volatile-organic-compound (VOC)-contaminated Soil in an Industrial Estate in Eastern Thailand by PCR-DGGE Analysis

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ABSTRACT

The prolonged contamination and accumulation of volatile organic compounds (VOCs) in soil and groundwater around industrial estate areas can have a negative impact on human health and the environment. *In situ* bioremediation by microbial activities is one of the most effective ways to remove soil pollutants. In order to promote the bioremediation process by nutrient application, it is important to understand the microbial community in the affected area. In this study, the diversity of bacterial communities in VOC-contaminated soil collected from eight sites within an industrial estate in the Eastern part of Thailand was determined by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis. The bacterial diversities in soil samples were evaluated in three enrichment media: tryptic soy broth, nutrient broth, and half-strength nutrient broth. Primers targeting 16S rRNA gene fragments were used. A total bacteria count was also performed in order to determine whether the number of bacteria were sufficient for bioremediation. We found that enrichment of soil with tryptic soy broth resulted in the greatest bacterial diversity. The bacterial communities in VOC-contaminated soil consisted mainly of Gram-negative Proteobacteria including Gamma- and Beta-proteobacteria, with genus *Pseudomonas* being prominent, and a Gram-positive member of phylum Firmicutes which was identified as *Bacillus*. The bacterial counts in most samples were higher than 3 logCFU·g⁻¹, which were high enough for *in situ* bioremediation. The results from this study could be used when considering nutrient application to increase the efficiency of bioremediation.

Keywords: VOC contaminated soil, industrial estate, PCR-DGGE, bacterial community

1. INTRODUCTION

Volatile organic compounds (VOCs) are organic carbon compounds with a vapor pressure greater than 0.1 mm Hg at 20°C, excluding carbon monoxide, carbon dioxide, carbonic acid, metallic carbides or carbonates, and ammonium carbonate [1]. The functional groups of VOCs released from many industrial processes have been identified as alkanes, alkenes, alkynes, aromatics, oxygenated and substituted compounds [2]. The Map Ta Phut Industrial Estate (MTPIE), located in Rayong province, is one of the largest industrial estates in Thailand. It comprises many companies in the petrochemical industry, chemical and fertilizer industry, steel industry, utilities industry and oil refinery industry [3]. These industries are potential sources of VOC release into the air, soil and groundwater. A study has shown that VOC accumulation around the MTPIE area has had some negative effects on human health and the environment [4].

There are different ways to remove or neutralize organic pollutants from soil and groundwater, such as soil vapor extraction, air sparging, carbon absorption, bioremediation, thermal desorption, pump-and-treat, stabilization/solidification, vitrification, and land treatment [5]. Bioremediation is a biological process that is increasingly used in pollution management. It uses naturally occurring microorganisms to remove or neutralize pollutants from a contaminated area, and it is considered safe. It can be used at the site as *in situ* bioremediation, thus effectively reducing the spread of pollutants to other sites [6]. Studies have shown that VOCs in soil can be degraded by microorganisms. This degradation process is understood to be due to the microorganisms' release of extracellular enzymes that digest complex organic

molecules. The microorganisms subsequently use degraded VOC products as an energy and carbon source for cell synthesis. Bacteria are the predominant microorganisms in the bioremediation process of most hydrocarbon pollutants [7]. Hydrocarbon-degrading bacteria are reported to be in the phylum of Proteobacteria, Bacteroidetes, Firmicutes, and Acinobacteria [8].

A prior knowledge of species diversity within a bacterial community is helpful in developing an *in situ* bioremediation process since the nutrients to be applied to VOC-contaminated soil can be appropriately designed based on this information [9]. In this study, we aimed to identify the diversity of the bacteria in the VOC-contaminated soil from areas around the MTPIE through polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) analysis in order to provide information for nutrient application in *in situ* bioremediation.

2. MATERIALS AND METHODS

2.1 Soil Sampling

The VOC-contaminated soil samples used in this study were obtained from eight sites in the MTPIE in Rayong province, Thailand. The soil samples were collected by Geoprobe drilling technique at a depth of 2.0 to 2.5 m below the soil surface. Soil samples were packed in sealed plastic bags and kept on ice while being transported to the laboratory. Upon arrival at the laboratory, soil samples were then stored at 4°C in the dark.

2.2 Determination of Physicochemical and Microbiological Properties of Soil Samples

Soil samples were analyzed for their physicochemical properties, including soil type, pH, and VOC concentrations. Soil

textures were identified using the hydrometer method [10]. The soil pH values were measured in 0.01 M CaCl₂ solution with the ratio of 1:2.5 (w/v) [11] using a pH meter (Metrohm 713, Switzerland). Concentration of dichloroethene (DCE), one of the VOCs, was determined by a gas chromatograph (7890A gas chromatograph) and a 5975C inert XL mass spectrometer (Agilent Technologies, U.S.A.), according to the U.S. Environmental Protection Agency (EPA) method 8260B.

Soil samples were also examined microbiologically for the total viable count using pour plate technique in plate count agar (PCA), using a ten-gram portion of each sample to make 10-fold serial dilutions. Counts were observed after being incubated at 37°C for 48 h from three replicates and calculated as colony forming unit per gram (logCFU·g⁻¹).

2.3 Genomic DNA Extraction

The soil samples were subjected to microbial enrichment using three enrichment media: tryptic soy broth (TSB), nutrient broth (NB), and half nutrient broth (½NB). Each soil sample was added to 30 mL of each medium followed by incubation at 37°C for 48 h. The suspensions of each enriched soil sample were centrifuged at 12,000 rpm in a microcentrifuge (Hettich MIKRO200R, Germany) at room temperature for 5 min and cell pellets were collected. The total genomic DNA from each sample was extracted using a protocol modified from Duhamel et al. [12]. The cell pellets were resuspended in TENS buffer (50 mM Tris-HCl; 20 mM EDTA pH 8.0; 100 mM NaCl; 1% w/v SDS) containing 0.5 g glass beads, mixed and incubated at 70°C for 20 min. The cell pellets were then horizontally vortexed for 6 min with alternate cooling in ice three times in between. Glass beads and cell debris were removed through

centrifugation at 12,000 rpm at room temperature for 10 min, and the aqueous phase was transferred to a fresh 1.5-mL microcentrifuge tube. The supernatant was extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), then twice with chloroform/isoamyl alcohol (24:1). The supernatant was added to cold absolute ethanol and the precipitated DNA was collected by centrifugation at 13,000 rpm at 4°C for 15 min. After being washed with 500 µL of 70% cool ethanol, the DNA pellet was dried and resuspended in deionized water.

2.4 PCR-DGGE Analysis of Bacterial Community

The 16S rRNA gene fragments were amplified by PCR using the universal eubacterial primers GC-63f (5'CGCCCGC CGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGCAGGCCTAACACA TGCAAGTC3', forward primer, with 40 bases GC clamp attached to the 5' end) and 518r (5'ATTACCGCGGCTGCTGG3', reverse primer) [13]. The expected length of the amplified fragment was 455 bp. The PCR mixture contained 2.5 µL of 10× KOD-Plus-Neo buffer, 1.5 mM of MgCl₂, 0.2 mM of dNTPs, 0.3 µM of each primer, 1U of KOD-Plus-Neo DNA Polymerase (TOYOBO, Japan), and 3% of dimethyl sulfoxide (DMSO). Then deionized water was added to the final volume of 25 µL. PCR conditions were performed using a thermal cycler (PowerPac, USA), with the conditions set as follows: initial denaturation at 94°C for 2 min, 30 cycles of 98°C for 10 s, 55°C for 30 s, 68°C for 30 s, with a final extension at 68°C for 7 min.

DGGE analysis was performed using a Dcode™ Universal Mutation Detection System (Bio-Rad, USA). PCR products were loaded onto 10% (w/v) polyacrylamide gels

with a denaturing gradient ranging from 25% to 50% (prepared from 100% denaturant gels containing 7 M urea and 40% (v/v) formamide). The electrophoresis was run at a constant voltage of 100 V for 360 min in 1× TAE buffer at 65°C. The gels were stained with 0.5 µg/mL ethidium bromide solution and visualized on a UV transilluminator (Vilber Lourmat VO36685, France).

The clear bands that appeared in the majority of the samples were excised from the gel and incubated overnight at 4°C in sterile distilled water. The excised bands were reamplified using the forward primer without a GC clamp (63f) and purified using a GF-1 AmbiClean Kit (Vivantis, USA). The reamplified products were sequenced by using the BigDye® Terminator v3.1 cycle sequencing kit's robust (performed by First BASE Laboratories, Malaysia). The similarity analysis of the partial 16S rRNA gene sequences were compared to the sequence deposited in GenBank using the BLASTN function. The sequences in this study were submitted to the DDBJ nucleotide database (accession numbers LC063678 to LC063690).

2.5 Analysis of DGGE Profiles

The DGGE patterns were analysed using the UV transilluminator Genesnap program (Genetool version 3.02.00 SynGene, UK). Clustering was compared using a pairwise similarity matrix calculated with the Dice's coefficient and the unweighted pair group method with arithmetic (UPGMA) method. The bacterial community structures of the DGGE profiles were determined based on the peak height values from each sample, which were analysed using the Excel program. The various biodiversity indices of VOC-contaminated soil were calculated in terms of the Richness (S) which determined the total number of distinct bands in each

lane from the DGGE patterns [14]. The Shannon index (H') [15] was also calculated on the basis of the number and intensity of bands present on the DGGE pattern, according to the following equations:

$$H = - \sum P_i \ln P_i = - \sum (n_i/N) \cdot \ln(n_i/N)$$

where P_i is the bands intensity to the sum of all peak height, calculated as follows: $P_i = n_i/N$, while n_i is the peak intensity of each band and N is the sum of the peak density of all bands in a lane.

2.6 Identification of Bacterial Isolates

Representatives of bacterial colonies grown on PCA were examined for the colony morphology, oxidase reaction and Gram reaction. Gram-negative bacteria were identified using the API 20 NE system (bioMerieux, France). Assimilation tests were read after 24 and 48 h and the results were analyzed using apiweb version 7.0. Gram-positive isolates were subjected to DNA extraction procedure (2.3) and PCR amplification (using primers 63f and 518r under the conditions listed above (2.4)). PCR products obtained from amplification of 16S rRNA gene from the isolates were identified by 16S rRNA gene sequencing and compared to sequence homology using BLASTN program in GenBank.

3. RESULTS AND DISCUSSION

3.1 Physicochemical and Microbiological Properties of Soil Samples

The physicochemical characteristics of the soil samples are summarized in Table 1. Soils from these industrial sites were mostly of clay type, which indicated that the soil has a high capacity for VOC adsorption [16]. The pH levels of most soil samples were slightly acidic, although a few samples were highly acidic (pH ranged 4.4 - 4.5; sample S2,

S3 in Table 1). The clay minerals of acidic soil play an important role in VOC adsorption [17]. Textural clay can adsorb organic compounds strongly through polyvalent cation interaction with organic matter. This ability increases the rate of VOC accumulation [18]. Some microbes are capable of removing organic compounds in an acidic environment [19]. The chemical analysis indicated that half of the soil samples were

contaminated with dichloroethene (DCE). DCE was also detected in groundwater around the MTPIE areas in a previous study [20]. The bacterial numbers in most samples were above 3 logCFU·g⁻¹. These were sufficient numbers for bioremediation according to Suthersan [21] and this condition can be used in contaminated area that as the previous report [22].

Table 1. Physicochemical properties and total bacteria counts of the soil samples.

site of sampling	pH	soil type	concentration of dichloroethene (ppm)	bacteria count (logCFU·g ⁻¹)
S1	6.4	clay	< 5	4.91
S2	4.4	clay	200	3.11
S3	4.5	clay	< 5	2.84
S4	6.4	clay	300	6.10
S5	6.4	clay	< 5	4.07
S6	6.1	sandy clay	250	4.21
S7	6.6	clay	< 5	3.74
S8	5.9	clay	50	3.19

3.2 Enrichment Media Selection

Among the three enrichment conditions (TSB, NB and ½NB), soil enriched with TSB medium was found to yield the highest numbers and intensity of DNA bands pattern of the DGGE profiles in comparison with in NB and ½NB media. TSB has also been previously reported to better support growth of many fastidious bacteria than NB [23]. Therefore, the TSB-enriched soil samples were selected for PCR-DGGE biodiversity analysis.

3.3 Bacterial Diversity by PCR-DGGE

From the DGGE profiles obtained from TSB-enriched soils (Figure 1), thirteen dominant bands were sequenced and analyzed with BLASTN analysis. Sequences of the DNA samples were analyzed against reference sequences in the database, and

their relatedness is shown in the neighbor-joining phylogenetic tree of 16S rRNA gene (Figure 2). The sequence analyses showed similarities to bacteria in genus *Pseudomonas*, *Salmonella*, *Citrobacter*, *Delftia*, *Enterobacter*, *Stenotrophomonas* and *Bacillus*. From 13 bands, about 69.23% of the selected bands were affiliated with Gammaproteobacteria and 23.08% were grouped into Betaproteobacteria. The Firmicutes phylum was only detected from one site and contributed 7.69%. The results from our study, together with those of other researchers, suggest that Proteobacteria and Firmicutes could play a significant role in the bioremediation process [24]. Bacteria in genus *Pseudomonas* have been shown to exist widely in contaminated soil and groundwater, and it is commonly utilized for biodegradation [25]. *Bacillus* spp. have also been reported

to have some roles in the bioremediation process, and they were more tolerant than many other bacteria to high levels of hydrocarbon pollutants in soil [26]. Moreover, bacteria in genera *Pseudomonas* and *Bacillus*

have been reported to be the dominant microorganisms that were involved in transformation of volatile *cis*- and *trans*-DCE compounds in soil and wastewater [27].

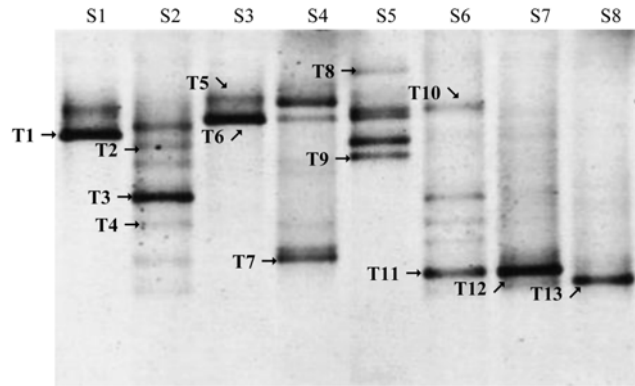


Figure 1. DGGE profiles of bacterial communities under the enrichment with tryptic soy broth (TSB).

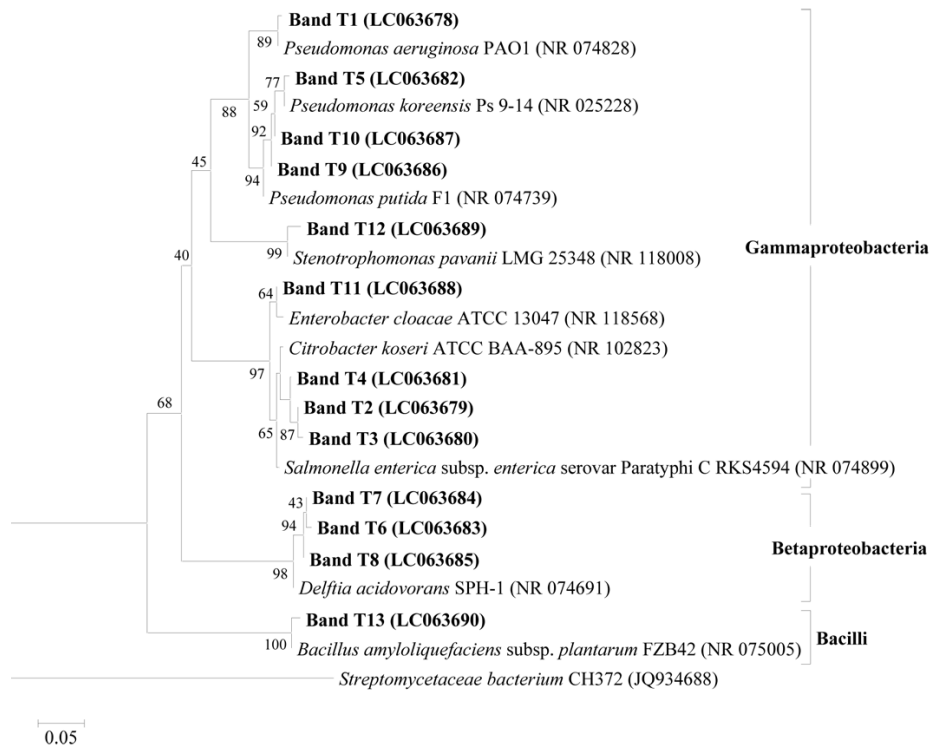


Figure 2. Neighbor-joining phylogenetic tree of 16S rRNA gene from the soils enriched with TSB medium. The bootstrap analyses with 1,000 replicates were shown at branch points. *Streptomycetaceae bacterium* CH372 was chosen as an out group.

The diversity of bacterial communities was indicated by the values of the Shannon index (H') and richness (S) observed from the DGGE profiles obtained from soil samples enriched with TSB (Table 2). The indices reflect the number of DGGE bands and the degree of the microbial community [28]. The genera detected at site S6, where greatest diversity was observed, contained *Pseudomonas*, *Salmonella*, *Citrobacter*, *Delftia* and *Enterobacter*. Interestingly, at site S3, which had very limited bacterial diversity, DCE was also found to be lower than the detection limit (<5 ppm), and the total viable count was low (Table 1). According to Thavamani et al. [29], the organic compound levels in contaminated soil are proportionally related to bacterial diversity. Although this was true in the case of S3 sample, it could not be generalized because the DCE levels were not necessarily correlated with the bacteria counts in other soil samples (S1, S5 and S7).

Table 2. Comparison of biodiversity indices from VOC contaminated soil in TSB medium by the Shannon index and their Richness.

sites	the Shannon index (H')	richness (S)
S1	0.826	3
S2	1.450	6
S3	0.605	2
S4	1.282	4
S5	1.047	3
S6	2.074	10
S7	1.634	7
S8	1.483	6

The dendrogram generated from UPGMA analysis is shown in Figure 3. The analysis showed three distinct clusters of DGGE profiles of the soil samples enriched with TSB. Soil bacteria in sites S7 and S8 were different from the other soil bacterial communities. The genera of

Stenotrophomonas and *Bacillus* were only present at sites S7 and S8, respectively. They were not detected in the other sites. The clusters of sites S3, S4, and S5 consisted mostly of *Pseudomonas* and *Delftia*, while the clusters of sites S1, S2, and S6 consisted mostly of *Pseudomonas*, *Salmonella*, *Citrobacter*, and *Delftia* species. The distinct bacterial communities observed in site S7 and S8 were correlated to the sampling locations (Figure 4).

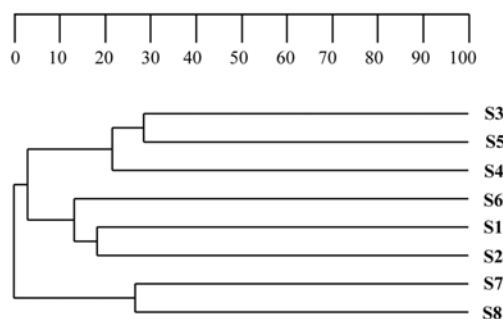


Figure 3. UPGMA dendrogram of DGGE profiles of the bacterial communities in soil samples obtained from different sampling sites (S1-S8).

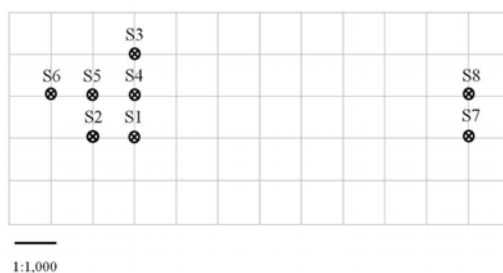


Figure 4. Diagram of soil sampling locations.

3.4 Identification of Bacterial Isolates

Bacterial isolates obtained from PCA were analyzed using API 20 NE and 16S rRNA analyses. The isolates were screened for their Gram reactions. The Gram-negative isolates were first identified by API 20 NE and confirmed by 16S rRNA gene analysis. API 20 NE identified these isolates as *Pseudomonas fluorescens* with 99.9% similarity

to the reference strains in the API database, and the sequences of 16S rRNA gene were identified as *Pseudomonas koreensis* with 99% similarity to the reference sequences (strain Ps 9-14). The discrepancy in species identification is not unusual; however, both 16S rRNA gene sequencing and API 20 NE generally give agreeable results at the genus level [30]. For the gram-positive isolates, they were identified by 16S rRNA gene sequencing. The results showed that the isolates were closely related to the phylum Firmicutes and belonging to species *Bacillus cereus*, *B. aryabhatai* and *B. siamensis*.

4. CONCLUSIONS

In this study, bacteria communities in VOC-contaminated soil from areas around Map Ta Phut Industrial Estate, one of the largest industrial estates in Thailand, were identified using PCR-DGGE. The microorganisms found in the soil were members of genus *Pseudomonas*, *Salmonella*, *Citrobacter*, *Delftia*, *Enterobacter*, *Stenotrophomonas*, and *Bacillus*. With the total number of bacteria counts in most samples being sufficient, the knowledge of species within the bacterial communities in VOC-affected areas would lead to appropriate nutrient formulation for the purpose of *in situ* bioremediation of the VOC-contaminated soil.

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