# Comparison of genomic DNA extraction methods for the next-generation sequencing analysis of bacterial community in the activated sludge after exposing to 1,3-butadiene

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## Abstract

The effective DNA extraction method is essential for an investigation of environmental microbial communities using next-generation sequencing (NGS).In this study, two DNA extracting protocols were evaluated based on yield and purity of the DNA. While an Al-precipitation method gave higher DNA yield, a commercial soil DNA extraction kit gave higher DNA purity and therefore selected as a suitable extracting protocol. This study also investigated effects of 1,3-butadiene (1,3-BD) exposure on bacterial community in activated sludge samples (designated as SL2 and SL4) collected from 2 different wastewater treatment facilities of petrochemical industry. While SL2 represented a bacterial community with no prior exposure to 1,3-BD and/or toluene, SL4 represented a bacterial community with prior exposure to both compounds. The sludge samples were enriched with 100 ppm of 1,3-BD (contributing 8,600 ppm of toluene as a solvent) for 6 weeks. The sludge samples exposed with only toluene were also conducted in parallel. Changes in microbial community profile was monitored based on 16s rDNA using next-generation sequencing platform. The sequences were assigned to the phylum as well as lower levels to represent the relative microbial abundance in each sample. Before an enrichment (Week 0), *Proteobacteria* (*Alpha-* and *Beta-proteobacteria*) were the dominant bacterial populations in both samples whereas *Firmicutes* was found only in SL2. After an enrichment with toluene, *Firmicutes* (*Bacilli*) was found to be the dominated taxa in SL2 while *Proteobacteria* (*Alpha-* and *Beta-proteobacteria*) decreased over time. In case of 1,3-BD exposure, *Proteobacteria* increased in both samples. Taxonomic classification into class level indicated that *Beta-proteobacteria* (mainly *Burkholderiales*) was the highest dominated bacterial populations in both samples exposed with 1,3-BD suggested that *Burkholderiales* may involve with degradation of 1,3-BD or its metabolites. An understanding on the bacterial community in toxic waste as well as how they evolve after an exposure can be applied further for selection of remediation solutions and environmental management.

Keywords: 1,3-Butadiene, Activated sludge, Bacterial community

## Introduction

The activated sludge system is a biological process widely used for wastewater treatment. Activated sludge consists of diverse bacterial communities responsible for the removal of various organic pollutants, toxicants and xenobiotics. Due to the high bacterial diversity, the conventional method such as culturing on media and denaturing gradient gel electrophoresis (DGGE) cannot fully represent the complexity and diversity of the bacterial community. Next-generation sequencing is the recent advanced technique widely used for exploring microbial structure with higher efficiency and resolution (Na et al., 2016; Keshri et al., 2015; Saunders et al., 2015; Giampaoli et al., 2014). For a study using NGS, the polymerase chain reaction (PCR) technique is an early important step used for a construction of DNA libraries. Good quality and high quantity of the DNA template are an essential requirement. During the extraction of DNA, the interfering compounds such as humic acid in the natural sources can be co-extracted and thus affected to the DNA recovery and reliability of the PCR amplification (Zielinska et al., 2016; Fortin et al., 2004). Therefore, the effective extraction and purification of DNA from different environmental samples are crucial for environmental genomic analyses. Several extraction procedures based on physical disruption, chemical lysis and enzymatic lysis have been reported for the isolation and purification of soil DNA samples. Many commercial DNA extraction kits are currently available and also widely used due to their high efficiency and simple extraction protocol. The suitable extraction method must be carefully selected based on DNA yield and purity.

1,3-butadiene (1,3-BD) is one of the 189 hazardous air pollutants under the 1990 U.S. Clean Air Act Amendments (CAAA). The International Agency for Research on Cancer (IARC, 1999), National Toxicology Program (NTP, 1984) and Environmental Protection Agency (USEPA, 1970) all classify 1,3-BD as a human carcinogen. It can be emitted into the atmosphere by various sources including combustion of fuels, during the production/storage/use/ transportation as well as being released from the waste treatment process. The Pollution Control Department (PCD) of Thailand sets the threshold level of 1,3-BD in air released from a waste treatment process of chemical manufacturing facility at ≤ 5 $mg/m^3$ (2.2 ppm). Although 1,3-BD is not expected to persist in atmosphere because of its high volatility and high reactivity, its contamination can contribute serious problems to environment as well as public health. Chou and Lu (1998) reported the use of biotrickling filter with pig manure compost and activated sludge to eliminate 1,3-BD in an air stream. There was, however, no detail information on the bacterial profile of the system. Analysis of bacterial community and their diversity in activated sludge is useful for improving of treatment efficiency as well as to understand the role of each bacterial group involving in waste treatment process.

In this study, two activated sludges were collected from different wastewater treatment systems. The genomic DNA were extracted by using two different protocols in order to compare the DNA extraction efficiency and purity. A suitable protocol was selected for preparation of DNA library. Before and after exposing to 1,3-BD, the bacterial community profile in the activated sludge was monitored using the next-generation sequencing platform.

**Objectives**

1. To evaluate 2 different DNA extracting protocols on yields and quality of the DNA
2. To investigate effects of 1,3-BD exposure on bacterial community in the activated sludge

**Scope of research**

1. Three activated sludge samples were collected from different wastewater treatment systems and one of them is a synthetic rubber manufacturing facility where 1,3-BD was used in the production process.
2. Research duration: January 2018 – October 2018

**Materials and Methodology**

1. Chemical and activated sludge samples collection

An analytical grade 1,3-BD (20% w/v in toluene) was purchased from Sigma-Aldrich (Queenstown, Singapore). Activated sludge samples (designated as SL1, SL2 and SL4) were collected from different wastewater treatment facilities and were kept at -20°C before use.

2. Effect of extracting protocols on yield and purity of the DNA

Genomic DNA was extracted from the activated sludge samples (SL1 and SL2) using 1) commercial genomic extraction kit for soil (Norgen Biotek, Canada) and 2) a protocol using Al-precipitation reported by Dong et al. (2006). Al-precipitation was employed to remove humic acid which can inhibit the activity of polymerase as well as restriction endonuclease (Wilson, 1997). The yield and purity of the DNA obtained were evaluated photometrically. Additionally, quality of the genomic DNA was evaluated by PCR amplification. The 16s rDNA fragment was amplified by touchdown PCR using the universal primers 27F and 1492R (Lane, 1991) with various DNA template volumes (0.25, 0.5, 1, 2, and 3 μL). The PCR products were checked using agarose gel electrophoresis.

3. An exposure with 1,3-BD

Changes in microbial community profile was monitored after exposing the activated sludge samples to 1,3-BD. Twelve grams of activated sludge samples (SL2 and SL4) were placed in a serum bottle containing 18 mL of sterile mineral salts medium ($KH_2PO_4$, 0.91g/L; $K_2HPO_4$, 0.40 g/L, $Na_2HPO_4$ $H_2O$; 2.39 g/L; $KNO_3$, 2.96 g/L; $(NH_4)_2SO_4$, 1.97 g/L; $MgSO_4$ $7H_2O$, 2.00 g/L; $FeSO_4$ $7H_2O$, 0.20 g/L; $NaHCO_3$. 0.50 g/L; $MnSO_4$ $H_2O$, 0.54 g/L; $ZnSO_4$ $7H_2O$, 0.04 g/L; $CaCl_2$, 2.26 g/L; $Na_2MoO_4$ $2H_2O$, 1.00 g/L; $CoCl_2$ $6H_2O$, 0.04 g/L; $Na_2EDTA$ $2H_2O$, 8.86 g/L) supplemented with 100 ppm of 1,3-BD (contributing 8,600 ppm of toluene as a solvent). The samples were incubated at 30°C, 200 rpm for 6 weeks. For comparison, similar experiment set up supplemented only toluene (at 8,600 ppm) was conducted in parallel.

4. Genomic DNA extraction, amplification and sequencing

During an enrichment of activated sludge samples (SL2 and SL4) with 1,3-BD for 6 weeks, genomic DNA from samples at week 0, 2, and 6 were extracted by using a suitable extracting protocol selected from the previous experiment. Purified genomic DNA was used for constructing of PCR library of the 16S V3–V4 region by using 16 amplicon forward primer (5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 16 amplicon reverse primer (5’-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3’). The amplification was performed in 80 µL reaction volume with the initial denaturation at 98°C for 2 min 30 s, 25 cycles of denaturation, annealing and elongation at 98°C for 10 s, at 55°C for 30 s and 72°C for 20 s, respectively, with a final extension step at 72°C for 2 min. The PCR product (550 bp) was and then purified using HiYield™ Gel/PCR DNA Fragments Extraction Kit (RBC, Taiwan). Genomic DNA concentration as well as the amplified PCR fragment were quantified using a Nanodrop2000c spectrophotometer (ThermoScientific, Wilmington, USA). The libraries were sequenced using MiSeq sequencer (Illumina) and the nucleotide sequence reads were aligned to a reference sequence with bioinformatics software. Operational taxonomic units (OTUs) were clustered at the 0.03 dissimilarity cut-off levels. The relative abundances (%) of single OTUs were calculated for each sample by comparing the number of total sequences assigned to a specific taxon against the number of total obtained sequences and were used to generate bar plots of bacterial diversity. BLAST database searching on NCBI was used to analyzed the bacterial sequences before and after the enrichment cultivation.

## Results and discussions

1. Effect of extracting protocols on yields and quality of the DNA

Genomic DNA were extracted from the activated sludge samples (SL1 and SL2) using 1) commercial genomic extraction kit for soil and 2) a protocol using Al-precipitation. Table 1 indicated that DNA yields obtained from Al-precipitation protocol were higher than those obtained from a commercial extraction kit in most cases. In case of Al-precipitation protocol, higher purity was observed when pH of 6.5 was used. This result agreed with the report by Cheng et al. (2004) that the efficiency of aluminium sulfate in humic acid removal was highest (about 90%) at pH 6 and decreased slowly to 80% at pH 7.Despite lower DNA yield, the commercial soil DNA extraction kit showed higher DNA purity than the Al-precipitation.

**Table 1** Optical density of the extracted DNA ($A_{260nm}$) and contaminated humic substances ($A_{340nm}$). SL1 = Sludge sample No.1; SL2 = Sludge sample No.2; K = genomic DNA extracted using commercial kit; D 6.5 and D 7 = the genomic DNA extracted by Al-precipitation reported by Dong et al. (2006) at pH 6.5 and 7, respectively; Positive control = genomic DNA extracted from bacterial cells using a commercial genomic DNA extraction kit

| Sample | | A230 | A260 | A280 | A320 | A340 | Concentration (µg/mL) | Purity (A260/A280) |
|---|---|---|---|---|---|---|---|---|
| **SL1** | SL1-K | 0.222 | 0.029 | 0.019 | 0.013 | 0.011 | 0.80 | 2.67 |
| | SL1-D6.5 | 0.035 | 0.033 | 0.021 | 0.011 | 0.010 | 1.10 | 2.20 |
| | SL1-D7 | 0.020 | 0.023 | 0.016 | 0.009 | 0.009 | 0.70 | 2.00 |
| **SL2** | SL2-K | 0.273 | 0.030 | 0.019 | 0.008 | 0.007 | 1.10 | 2.00 |
| | SL2-D6.5 | 0.074 | 0.047 | 0.039 | 0.025 | 0.023 | 1.10 | 1.57 |
| | SL2-D7 | 0.112 | 0.061 | 0.053 | 0.030 | 0.027 | 1.55 | 1.35 |
| **Positive control** | | 0.029 | 0.025 | 0.017 | 0.011 | 0.010 | 0.70 | 2.33 |

DNA concentration and DNA purity were calculated using the following formula;

DNA concentration (µg/mL) = (A260 – A320) × dilution factor × 50 µg/mL

DNA purity (A260/A280) = (A260 – A320)/(A280 – A320)

Positive controls were included in order to check that the PCR conditions used can amplify the target sequence effectively. The absence of 16s rDNA bands (expected size of approximately 1.5 kb) (Figure 1) at a template volume ≥ 1 µL, in which the positive control still yielded PCR products, suggested the presence of biological inhibitors (e.g. humic acids) in the soil DNA obtained from both protocols. However, at a template volume of 0.5 µL in which the inhibitors were diluted, the PCR products could be observed in all DNA templates except SL2-D7 which exhibited lowest DNA purity of 1.35 (Table 1). Considering the fact that DNA extracted from the commercial kit constantly yielded PCR products when proper dilutions were applied, this protocol was considered most suitable and was selected for further use.

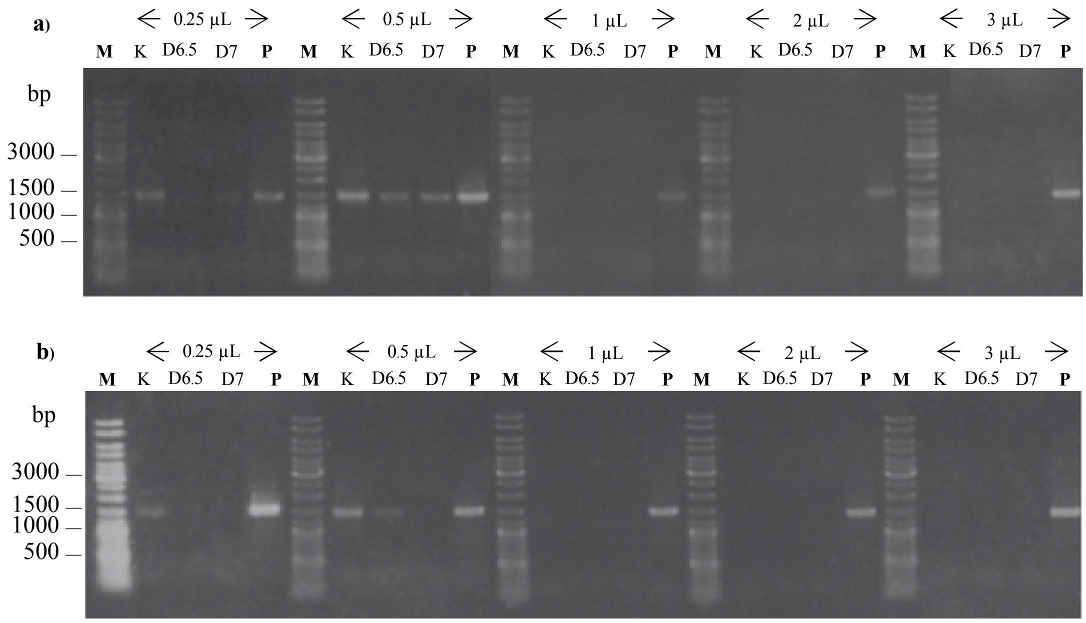


**Figure 1** Result of PCR amplification using DNA extracted from the sludge samples with different protocols; K = genomic DNA extracted using commercial kit, D6.5 = genomic DNA extracted by Al-precipitation at pH 6.5 and D7 = genomic DNA extracted by Al-precipitation at pH 7 (Dong et al., 2006). (a) PCR products from SL1 sample, (b) PCR products from SL2 sample with different DNA template volume per reaction (20 µL). The template volume used was indicated above the lane. M is molecular marker (Thermo Scientific, USA). P is a positive control (genomic DNA extracted from cells of *Bacillus licheniformis*). These gels were made using 0.8% agarose with GelRed™ staining.

2. Genomic DNA extraction, PCR library preparation and sequencing

The classification at the phylum level for each activated sludge bacterial community was summarized in Fig. 2. Before an exposure (Week 0), *Proteobacteria* was the dominant bacterial group, constituting 30-45% of the bacterial population in both activated sludge samples. This is in agreement with the bacterial communities observed in activated sludges from various wastewater treatment systems (Xu et al., 2018; Isazadeh et al., 2016; Wang et al., 2012; Xia et al., 2010) which were dominated by the phylum *Proteobacteria*. *Planctomycetes* (3-16%) and *Chloroflexi* (3-11%) found as subdominant groups in this study were previously reported in bacterial communities of an activated sludge (Wagner et al., 2002). Although *Firmicutes* was detected only in SL2 in this study, they were found in high abundance in several activated sludge samples reported in literatures (Ye et al., 2011; Kwon et al., 2010; Xia et al., 2010).

After an enrichment with toluene (Figure 2a), *Planctomycetes*, *Chloroflexi* and *Chlorobi* tended to decrease over time while *Actinobacteria* and *Candidatus Saccharibacteria* (Candidate division TM7) increased continuously. *Actinobacteria* was reported as the dominant member (accounting for 60%) in soil after an exposure with toluene (Olapade et al., 2015) whereas *Candidatus Saccharibacteria* (Candidate division TM7) was reported as the dominant bacterium responsible for carbon uptake from toluene in agricultural soil (Luo et al., 2009). In SL2, *Firmicutes* (*Bacilli*) was found to be the dominated taxa during toluene exposure while *Proteobacteria* (*Alpha*- and *Beta-proteobacteria*) decreased over time (Figure 2a). This observation is in agreement with the fact that several *Bacillus* strains have been reported for their ability to degrade toluene (Olapade et al., 2015).

In case of 1,3-BD exposure, *Proteobacteria* steadily increased in both samples (Figure 2b). Taxonomic classification into class level of *Proteobacteria* indicated that *Alpha-proteobacteria* (mainly *Rhizobiales*) and *Beta-proteobacteria* (mainly *Burkholderiales*) were the most abundant classes in both samples (Figure 3). Literature related with the bacterial profile during 1,3-BD biodegradation is sorely lacking. 1,3-BD was only reported once in the biofilter using pig manure compost and activated sludge (Chou and Lu, 2011). The fact that *Beta-proteobacteria* (mainly *Burkholderiales*) was the highest dominated bacterial populations in both samples exposed with 1,3-BD (comparing to those exposed to toluene alone) suggested that *Burkholderiales* may involve with degradation of 1,3-BD or its metabolites.

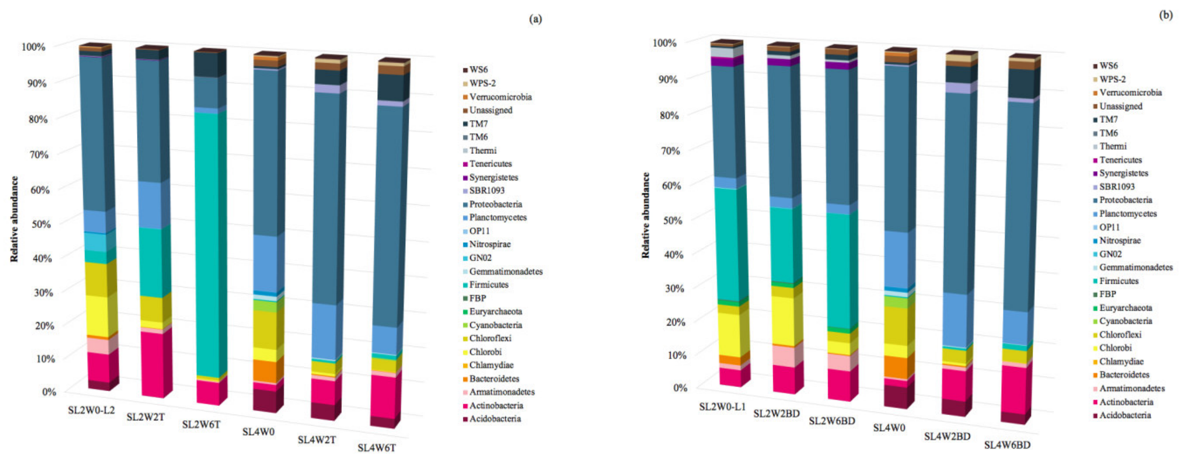


**Figure 2** (a) The relative abundance of activated sludge samples (SL2 and SL4) before (SL2WK0-L2 and SL4WK0) and after exposure to toluene for 2 (SL2WK2T and SL4WK2T) and 6 weeks (SL2WK6T and SL4WK6T); (b) The relative abundance of activated sludge samples (SL2 and SL4) before (SL2WK0-L1 and SL4WK0) and after exposure to 1,3-BD for 2 (SL2WK2BD and SL4WK2BD) and 6 weeks (SL2WK6BD and SL4WK6BD). The relative abundance is presented in terms of a percentage of the total bacterial sequence in sample.

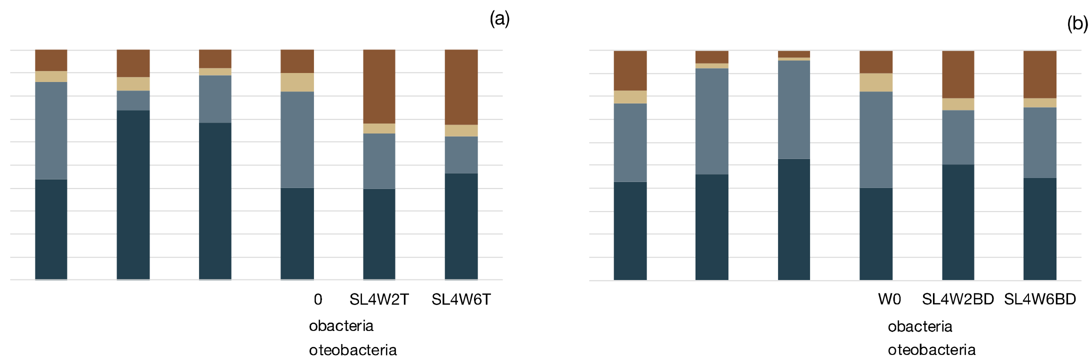


**Figure 3** The community composition of the phylum *Proteobacteria* in the activated sludge samples (SL2 and SL4). (a) The relative abundance of activated sludge samples before (SL2WK0-L2 and SL4WK0) and after exposure to toluene for 2 weeks (SL2WK2T and SL4WK2T) and 6 weeks (SL2WK6T and SL4WK6T). (b) The relative abundance of activated sludge samples before (SL2WK0-L1 and SL4WK0) and after exposure to 1,3-BD for 2 weeks (SL2WK2BD and SL4WK2BD) and 6 weeks (SL2WK6BD and SL4WK6BD).

## Conclusions

The results from this study demonstrated that the microbial communities in activated sludge samples collected from 2 different wastewater treatment facilities responded differently towards an exposure to 1,3-BD and/or toluene. *Proteobacteria* was the dominant phylum in both samples (SL2 and SL4) whereas *Firmicutes* was found only in SL2. *Actinobacteria* and *Candidatus Saccharibacteria* (Candidate division TM7) were increased continuously in both samples when exposed to toluene. *Firmicutes* (*Bacilli*) was found to be the dominated taxa during toluene exposure only in SL2. During 1,3-BD exposure, *Proteobacteria* (*Alpha-* and *Beta-proteobacteria*) was the most abundant phylum in both samples. Within *Proteobateria*, *Beta-proteobacteria* (mainly *Burkholderiales*) was the highest dominated bacterial populations in both samples exposed with 1,3-BD (comparing to those exposed to only toluene) suggested that this group of bacteria may associate with the degradation of 1,3-BD or its metabolites.

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