

Original Article

Evaluation of enhanced bioremediation for soils contaminated with used lubricating oil in soil slurry system

Naruemon Meeboon¹ and Suppasil Maneerat^{2*}

¹ Department of Biological Innovation, Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University, Tha Ngio, Mueang, Nakhon Si Thammarat, 80280, Thailand

² Biotechnology for Bioresource Utilization Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand

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Abstract

This study was conducted to evaluate the biodegradability of used lubricating oil (ULO) in soil slurry system using bioaugmentation and biostimulation approaches. Bioaugmentation with SC9 consortium isolated from ULO-contaminated soil and biostimulation with biosurfactant obtained from *Acinetobacter calcoaceticus* subsp. *anitratus* SM7 as well as nutrients were investigated. Soil concentration, initial pH, type and concentration of nutrients, and inoculum size of SC9 consortium affected by the rate of microbial degradation of ULO in soil slurry system. Maximal degradation rate of ULO (63.3%) was obtained when SC9 consortia was incubated under the optimized conditions for 7 days. The maximum biodegradation and bacteria growth were 88.6% and 10.9 log CFU ml⁻¹ in unsterilized soil slurry supplemented with SC9 consortium and crude biosurfactant after 30 days of incubation. Biosurfactant derived from *A. calcoaceticus* subsp. *anitratus* SM7 and/or SC9 consortium are significant factors in supporting indigenous microbial growth and added to soils to enhance degradation of ULO in soil slurry bioremediation. Therefore, both SC9 consortium and biosurfactant have potential for apply in soil slurry bioremediation of ULO and other hydrocarbons-contaminated soil.

Keywords: biodegradation, biosurfactant, consortium, soil slurry, used lubricating oil

1. Introduction

Billions of gallons of used lubricating oil (ULO) are generated every year in the world. ULO is considered to be hazardous waste materials. ULO contains heavy metals, aliphatic hydrocarbons, aromatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) that would contribute to chronic hazards including carcinogenicity (Dindar, Şağban, & Başkaya, 2016). Currently, ULO generated from mechanical workshops is becoming a visible problem in Thailand (Meeboon, Leewis, Kaewsawan, Maneerat, & Leigh, 2017; Pimda & Bunnag, 2012).

Bioremediation methods have been developed to support and increase the degradative activities of native microbial populations, thus allowing a reduction in time required for the expected bioremediation. In order to bioremediation of a contaminated soil to succeed, it is necessary to have favorable environmental conditions that foster microbial activity and contaminant biodegradation, such as soil matrix, sufficient nutrients, indigenous soil microbes and no inhibitors of microbial metabolism (Chikere, Okpokwasili, & Chikere, 2011).

Accordingly, this study was conducted to accelerate the biodegradation of ULO in soil slurry system by using consortium bacterial culture and biosurfactant. Bacterial diversity of SC9 consortium was characterized with PCR-DGGE. Factors affecting ULO degradation by SC9 consortium in soil slurry including soil concentration, inoculum size, nutrient source and initial pH of soil slurry were studied. In addition,

*Corresponding author
Email address: suppasil.m@psu.ac.th

potential application of crude biosurfactant produced by *Acinetobacter calcoaceticus* subsp. *anitratus* SM7 for enhancement biodegradation of ULO in soil slurry was also investigated.

2. Materials and Methods

2.1 Used lubricating oil (ULO)

ULO was obtained from garage around Prince of Songkla University, Thailand. It was used as a carbon source in mineral salt medium (MSM) composed of (g/l) K₂SO₄, 1.8; KH₂PO₄, 1.2; NH₂Cl, 4.0; MgSO₄·7H₂O, 0.2; NaCl, 0.1; FeSO₄·7H₂O, 0.1 (modified from Ijah & Ukpe, 1992).

2.2 Soil characteristics

Soil without ULO contamination was collected from orchards in Songkhla Province, Thailand. The soil samples were taken from a depth of 15 cm by digging with a sterile trowel and sieved through 4 mm mesh to remove gravel and plant debris. The soil samples were stored in sterilized containers and placed in a dark, dry area at room temperature (29±2 °C) to prevent the occurrence of photooxidation reactions.

Air dried soil samples were primarily characterized for pH (Back, Banwart, & Hassett, 1980), moisture content (Brady, 1974) and total hydrocarbon (Sharma et al., 1998). The type of soil, organic matter, total nitrogen and available phosphorus were analyzed by the Department of Soil Science, Faculty of Natural Resource, Prince of Songkla University.

2.3 Microorganisms and cultivation conditions

The SC9 consortium was previously isolated from ULO-contaminated soil collected from garages in Nakhonrithammarat Province, Thailand (Meeboon, 2008). Aliquots (50 µl) of the SC9 consortium stored at -20 °C were transferred to 5 ml of nutrient broth (NB, Himedia, India) and incubated in an orbital shaker at 200 rpm for 12-24 h at room temperature. A 1 ml aliquot of bacterial culture was transferred to 50 ml NB flask and incubated at the same condition as previously described. The cells were then harvested by centrifugation (6,000×g, 20 min, 4 °C), washed two times in 0.85% sterile saline before being resuspended in 0.85% sterile saline. From this bacterial preparation, a 10% (v/v) inoculum (7.0 log CFU ml⁻¹) was used in the following experiments.

2.4 Experimental design for ULO degradation

The influence of soil level, inoculum size, initial pH, nitrogen and nitrogen concentration on the ULO biodegradation were investigated. The SC9 consortium was transferred to 50 ml MSM medium containing 1% ULO as a carbon source following the previous work (Saimmai, Kaewrueng, & Maneerat, 2012) in a 250 ml flask. The initial pH of MSM medium was 7.0 and incubated at room temperature in an orbital shaker at 200 rpm for 7 days. To quantify the effect of soil levels on ULO degradation, five different sets of experiment by varying soil concentrations (0%, 5%, 10%, 15% and 20%, w/v) were performed. Determination the effect of inoculum size by varying their concentration from 5 to 25% (v/v) and effect of different initial pHs (5-9.5) for ULO degradation was also

studied. In order to investigate the effect of nitrogen source on ULO degradation, ammonium chloride (NH₄Cl), ammonium nitrate (NH₄NO₃), ammonium sulfate ((NH₄)₂SO₄) and urea were added to MSM medium and then conducted to determine the effect of nitrogen source concentrations (0, 2, 4, 6 and 8 g l⁻¹). The samples were analyzed for pH and total viable counts by spread plate technique on nutrient agar. The oil degradability was calculated based on weight loss method. Quantitative analysis of ULO in the supernatant was performed by extracting 50 ml culture broth twice with 50 ml hexane. The solvent layer was combined and dried with anhydrous Na₂SO₄. Then, the solvent was evaporated by vacuum rotary evaporator. Dry weight was determined, and the oil degradability was calculated based on weight loss (Shirai, Hanzawa, & Katusta, 1995) as follow:

$$\text{Weight loss (\%)} = \frac{(W_i - W_r)}{W_i} \times 100$$

where W_i and W_r are the initial and residual ULO weight, respectively. Each condition which gives the highest oil degradation was selected for further study on the bioremediation of ULO in soil slurry system.

The bioremediation of ULO in sterilized and non-sterilized soil slurry were investigated. Eight different experiments were designed (Table 1). The crude biosurfactant (35 mg) obtained from *A. calcoaceticus* subsp. *anitratus* SM7 (Phetrong, H-Kittikun, & Maneerat, 2008) was used to accelerate the degradation of ULO in soil slurry system. The soil samples were shaken at 200 rpm and incubated at room temperature and the samples were taken at 0, 7 and 30 days. All treatments were carried out in triplicates. The samples were analyzed for pH, total viable counts, weight loss and ULO compositions by GC-MS (Dąbrowska, Dabrowski, Biziuk, Gaca, & Namiesnik, 2003).

2.5 PCR amplification and DGGE analysis

SC9 consortium from stock solution (2%) were transferred to 5 ml of NB medium and incubated in an orbital shaker at 200 rpm at room temperature for 24 hours. Cells of SC9 consortium were extracted with the GB300 Genomic DNA Mini Kit (Geneaid biotech Ltd., Taiwan). These DNA extracts were used as templates for a PCR reaction. PCR universal primers 341F and 517R were used to amplify the V3 region of bacterial 16S rDNA (Muyzer & Smalla, 1998) following standard procedures described by Röling *et al.* (2002).

Table 1. Overview of microcosms set-up.

No.	Amendments	Microcosm set-up
1	Sterilized soil* with no amendments	SS
2	Sterilized soil with SC9 consortia	SS + SCI
3	Sterilized soil with crude biosurfactant	SS + CB
4	Sterilized soil with all amendments	SS + SCI + CB
5	Non-sterilized soil, No amendments	NSS
6	Non-sterilized soil, soil indigenous, SC9 consortia	NSS + SCI
7	Non-sterilized soil, soil indigenous, crude biosurfactant	NSS + CB
8	Non-sterilized soil, all amendments	NSS + SCI + CB

*Soil was sterilized at 121°C for 30 min. SS = sterile soil; NSS = non-sterile soil; SCI = SC9 consortia inoculum; CB = crude biosurfactant.

Bacterial community fingerprints were carried out by DGGE using a 12 h running time. Reverse primer 517R included a GC-rich tail sequence for fragment stabilization during DGGE. Each gel contained a linear gradient of the denaturants urea and formamide, increasing from 30% at the top to 60% at the bottom was applied to separate 16S rDNA fragments. Gels were stained in 1×TAE buffer containing 1×SYBR gold DNA gel stain (10 ml) and photographed. Pieces of the DNA bands were cut off the electrophoresis gels, re-amplified, and sequenced. Those sequences were evaluated by using the basic local alignment search tool to determine the closest relatives in the GenBank databases (<http://www.ncbi.nlm.nih.gov/BLAST/>).

2.6 Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. Statistical analysis was performed using Statistical Package for Science. (SPSS 10.0 for Window, SPSS Inc., Chicago, IC).

3. Results and Discussion

3.1 Soil characteristics

Characteristically selected soil sample belongs to loam (17.32% clay, 37.61% silt and 45.06% sand). Moisture content and pH of soil sample were 20% and 6.29, respectively. The organic matter content, total nitrogen and available phosphorous were 25, 1.3 and 0.54 g kg⁻¹, respectively (Table 2). Loam soil tends to have high nutrient retention, and organic matter, which may have high number of indigenous bacteria.

3.2 Effect of soil concentration on the biodegradation of ULO by SC9 consortium

The degradation of ULO showed considerable variation regarding to soil concentration. The results have clearly shown that soil concentration play an important role to hydrocarbons degradation. The optimal of soil concentration (10% of soil) showed the highest degradation of ULO about 50.5% (Figure 1a) and it also enhanced the growth of SC9 consortium. SC9 consortium grew and reached a maximum of 10.2 log CFU ml⁻¹ at 7 days. The degradation of ULO in 0%, 5%, 15% and 20% of soils was 40.0%, 22.3%, 17.1% and 16.9%, respectively.

The reason for the higher ULO degradation potential of SC9 consortium in 0% of soil than 5% of soil when incubated in MSM medium may be because of the population of SC9 consortium in 0% of soil (10.2 log CFU ml⁻¹) achieved higher count than 5% of soil (9.9 log CFU ml⁻¹). The SC9 consortium in MSM medium without soil have the high contraction with hydrocarbon compounds of ULO, but the reaction was reduced when added soil (5%) into the medium. That is because the hydrocarbon compounds of ULO bind to soil particles, and they are difficult to be removed or degraded (Erdogan & Karaca, 2011). The presence of soil more than 10% is clearly shown to have strong negative effect on ULO biodegradation and bacterial population. Therefore, the organic matter content may affect to ULO biodegradation in soil slurry process. The organic matter content is crucial to hydrocarbon adsorption/

desorption in soil (Chien, Chen, & Li, 2018). The organic matter in soil serves as a store house of carbon and energy as well as a source of other macronutrients such as nitrogen, phosphorus and sulfur. It is well known that soil application of organic residues accelerates removal of hydrocarbon from soil (Hamdi, Benzarti, Manusadzianas, Aoyama, & Jedidi, 2007). Organic materials present in the added soils (10%) must have supported the cell growth. The absorption of hydrocarbons by soil slurry may explain why hydrocarbon toxicity appeared sometime after contamination in the soil. Hydrocarbons are initially absorbed by organic matter in soil slurry. As organic matter mineralization and/or desorption processes occurred, hydrocarbon

Table 2. Physical and chemical characteristics of soil sample

Soil property	
Texture	Loam
- clay (%)	17.32
- silt (%)	37.61
- sand (%)	45.06
pH	6.29
Moisture content (%)	20
Organic matter (g kg ⁻¹)	25
Total hydrocarbon (g kg ⁻¹)	0
Total N (g kg ⁻¹)	1.3
Available P (g kg ⁻¹)	0.54

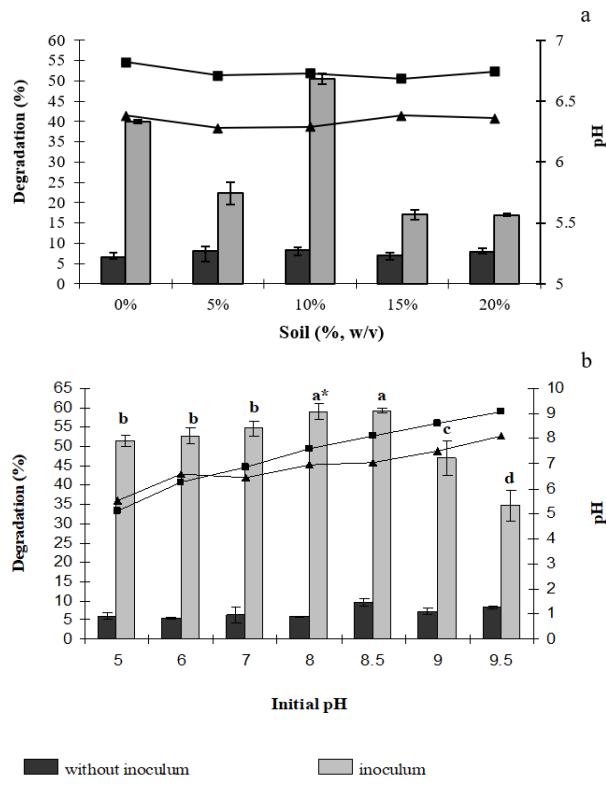


Figure 1. Effect of soil and initial pH on degradation of ULO (1%) in 50 ml MSM by SC9 consortium and pH changes after incubation at room temperature, 200 rpm for 7 days. Bars represent the standard deviation from three determinations.

*Different letters indicate significant differences ($p < 0.05$).

concentration in the aqueous increased, the toxic effect of hydrocarbons on microbial populations becoming more noticeable (Labud, Garcia, & Hernandez, 2007). The initial pH of the soil slurry was 7.0 and the final pH was 6.29. Decreasing pH of the culture medium after seven days of incubation is probably due to the release of proton ions formed and other metabolic products from microbial consortium during hydrocarbon degradation (Yu, Wong, Yau, Wong, & Tam, 2005).

3.3 Effect of initial pH on the biodegradation of ULO by SC9 consortium

The initial pH of the soil slurry had influence the degradation of ULO by SC9 consortium. The highest proportion of ULO was degraded by the SC9 consortium with 59.3% and 58.9% at pH 8.5 and 8.0, respectively. However, there was no significant difference in ULO degradation between these two treatments ($p>0.05$) (Figure 1b). At pH 8.5, the amount of SC9 consortium increased from 7.0 log CFU ml⁻¹ at the beginning to a maximum count of 11.5 log CFU ml⁻¹ after 7 days of incubation. The initial pH from 5 to 7 substantially increased biomass growth and ULO biodegradation percentage. The initial pH more than 8.5, dramatically decreased both biomass growth and ULO biodegradation, with a worse ULO biodegradation percentage (34.7%) at pH 9.5. Hydrocarbon degrading thrives well in the optimum degradation occurring under slightly alkaline or near neutral pH conditions (Abu & Dike, 2008). If pH stress membrane permeability and superoxide anion (O₂⁻) production were increased to high levels showing dramatic physiological changes. A few bacterial species have adapted to life at more acidic or alkaline extremes (Baatout, Leys, Hendrickx, Dams, & Margeay, 2007; Li, Hao, Sheng, & Xu, 2008).

3.4 Effect of inoculum size on the biodegradation of ULO by SC9 consortium

Inoculum size of SC9 consortium affected the degradation of ULO. As illustrated in Figure 2, an increase in the inoculum size significantly enhanced biodegradation efficiency of SC9 consortium ($p < 0.05$). The degradation of ULO with SC9 consortium was 55.2%, 57.1% and 58.4% at 15%, 20% and 25% of inoculum size, respectively. However, there was no significant difference in ULO degradation between these three treatments ($p>0.05$). To increase in cell concentration would decreasing lag period for growth of bacterial strain and increasing hydrocarbon degradation rate (Palanisamy *et al.*, 2014). Pathak, Kantharia, Malpani, and Madamwar (2009) have reported evaluation of inoculum size on naphthalene degradation by *Pseudomonas* sp. HOB1. Higher inoculum size resulted in maximum naphthalene degradation up to 100%. An uninoculated treatment loss of ULO was 6.4%. pH of all treatments dropped to around 6.24-6.61 from the initial pH of 7.0. The cell counts increased from 7.0 log CFU ml⁻¹ at the beginning of the study to maximum amount of 12.7 log CFU ml⁻¹ at 25% inoculum size.

3.5 Effect of nitrogen source and concentration on degradation of ULO by SC9 consortium

The effect of nitrogen source on degradation of ULO by SC9 consortium is shown in Figure 3a. The results showed that addition of ammonium sulfate instead of ammonium chlo-

ride gave the highest degradation of ULO about 65.2% after seven days (Figure 3a) ($p < 0.05$). The amount of SC9 consortium was also highest (11.4 log CFU ml⁻¹) when ammonium sulfate was used ($p < 0.05$). An understanding of nutrient status at a specific site is essential for a successful biodegradation of hydrocarbon. Since nitrogen is a key building block of proteins and nucleic acids, it is important to add a nitrogen source in contaminated soil for enhance the biodegradation rate of petroleum hydrocarbons (Acuña, Pucci, & Pucci, 2012; Hesnawi, & Adbeib, 2013). The degradation of ULO with ammonium chloride, ammonium nitrate and urea were 60.6%, 57.2% and 15.1%, respectively. However, there was significant difference in ULO degradation between these three treatments ($p < 0.05$). Altering the nitrogen source with organic nitrogen, urea did not play an important role in improving the removal efficiency of ULO. This might be excessive addition of urea may increase pH and toxic to bacterial consortium in soil slurry. Strong urea used disrupts bacterial cell membranes and denature membrane proteins that may increase pH in treatment with inoculum and extremely decrease the amount of SC9 consortium within seven days of incubation (Ratzke & Gore, 2018; Vyas & Dave, 2010).

Degradation of ULO increased with increasing ammonium sulfate concentration from 0 to 4 g l⁻¹ (Figure 3b). The results indicate that an increase in the concentration of ammonium sulfate would promote ULO degradation (Figure 3b). The maximum degradation efficiency of 63.3% by SC9 consortium was observed in the medium containing 4 g l⁻¹ ammonium sulfate (C:N ratio of 100:8). In the fertilized soil samples, the total amount of hydrocarbons degraded increased with increasing concentrations of nitrogen (Janaki, Thenmozhi, & Muthumari, 2016). Fangrui (2017) studied the influence of nitrogen concentration (1-5 g l⁻¹) on alkanes degradation of *Alcanivorax* sp. H34 in medium. This strain can grow best, and the growth reaches the maximum when supplement with 3 g l⁻¹ of NH₄NO₃ within 96 hours of incubation. Ammonium sulphate 4 g l⁻¹ stimulated microbial growth from 7.0 log CFU ml⁻¹ to 11.4 log CFU ml⁻¹ within 7 days and thus lead to extensive degradation of ULO in comparison to the control. Without ammonium sulfate no growth was observed in soil slurry treatment. However, the

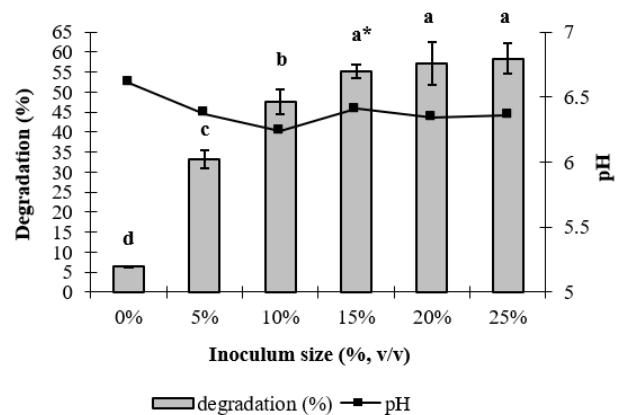


Figure 2. Effect of SC9 consortium inoculum size on degradation of ULO (1%) in 50 ml MSM containing 10% soil and pH changes after incubated at room temperature, 200 rpm for 7 days. Bars represent the standard deviation from three determinations. * Different letters indicate significant differences ($p < 0.05$).

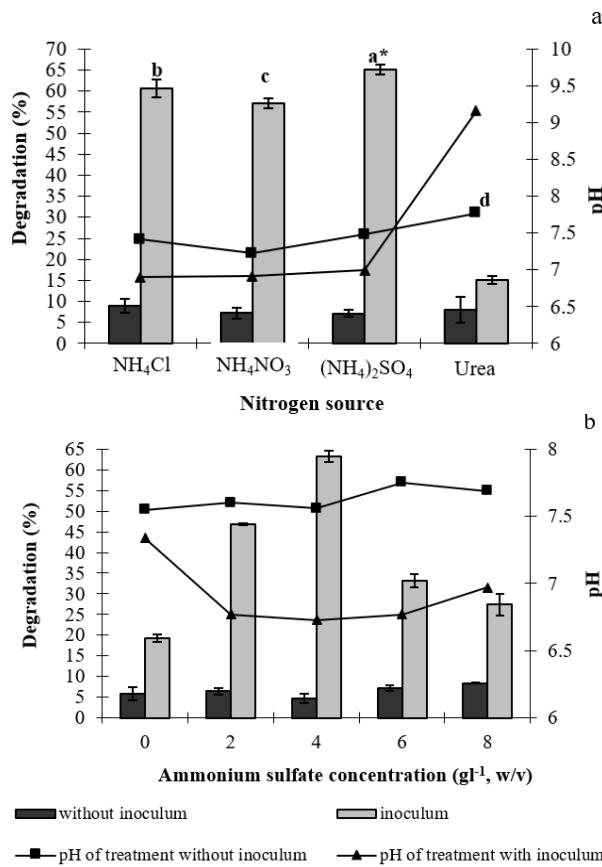


Figure 3. Effect of nitrogen source and ammonium sulfate concentration on degradation of ULO (1%) in 50 ml MSM initial pH 8.0 containing 10% soil and 15% SC9 consortium inoculum size and pH changes after incubated at room temperature, 200 rpm for 7 days. Bars represent the standard deviation from three determinations. *Different letters indicate significant differences ($p < 0.05$).

ULO degradation was markedly decreased when ammonium sulfate concentration higher than 4 g l^{-1} . Excessive ammonium sulfate concentrations in soil slurry system may inhibit ULO biodegradation activity (Dindar *et al.*, 2016; Mukred, Hamid, Hamzah, & Yusoff, 2008). From the obtained results can be concluded that high concentrations of ammonium sulfate had reduced the bacterial activity and subsequently can be lethal to bacterial cell. Biodegradation of ULO is a complex process that depends on the amount of bacteria present in each treatment.

3.6 Effect of bioaugmentation and biostimulation on ULO bioremediation in soil slurry system

In the present study, potential application of SC9 consortium (bioaugmentation) for enhance bioremediation of ULO-contaminated soil slurry was evaluated. Biostimulation (nitrogen source and crude biosurfactant) and natural attenuation were also investigated in soil slurry system. Our results demonstrated that SC9 consortium with crude biosurfactant (SS + SCI + CB) showed the highest activity to degrade ULO up to 77.26% at day 7 of experiment (Figure 4). However, the treatments of SC9 consortium together with crude biosurfactant and

indigenous microorganisms (NSS + SCI + CB) showed highest ULO degradation activity and bacterial growth reached to 88.6% and $10.9 \log \text{CFU ml}^{-1}$, respectively at day 30 of experiment (Figure 4, 5). These results suggested that crude biosurfactant could enhance dramatically the mass transfer of ULO thus allowing its increased conversion and degradation by both indigenous bacteria and inoculated SC9 consortium. ULO-contaminated soil supplemented with biosurfactants and hydrocarbonoclastic bacteria consortium could increase bioavailability of hydrocarbons and biodegradation process (Fitri, Ni'matzahroh, & Surtiningsih, 2016). Crude biosurfactant addition had positive effects suggesting these amendments were important to restore the bioavailability of essential micronutrients and thus the balance with the carbon content (Saeki, Sasaki, Komatsu, Miura, & Matsuda, 2009; Salihu, Abdulkadir, & Almusta-

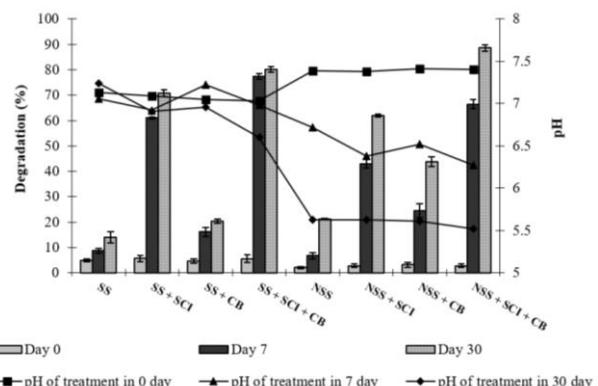


Figure 4. Effect of crude biosurfactant on the degradation of ULO (1%) in 50 ml MSM initial pH 8.0 containing 10% soil, 15% SC9 consortium inoculum size and 4 g l^{-1} $(\text{NH}_4)_2\text{SO}_4$ in sterilized and non-sterilized conditions and pH changes after incubated at room temperature, 200 rpm for 0, 7 and 30 days. Bars represent the standard deviation from three determinations.

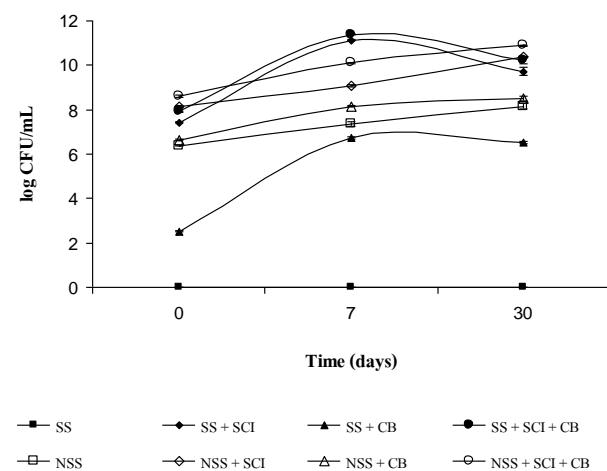


Figure 5. Total viable count after 30 days of degradation of ULO (1%) with 4 g l^{-1} $(\text{NH}_4)_2\text{SO}_4$ in 50 ml MSM medium initial pH 8.0, containing 10% soil and 15% SC9 consortium inoculum size and incubated at room temperature, 200 rpm for 0, 7 and 30 days.

pha, 2009). Biosurfactants affect bioremediation processes due to their efficacy as dispersion, remediation agent, environmentally friendly, biodegradable, less toxicity and non-hazardous (Rita de Cássia *et al.*, 2014). Ebadi, Sima, Olamaee, Hashemi, and Nasrabadi (2017) studied the ability of nutrient (biostimulation) and a surfactant-producing *Pseudomonas aeruginosa* consortium (bioaugmentation) to degrade a petroleum-polluted saline soil. The degradation efficiency of the microcosm of the consortium together with biostimulation was significantly greater than those measured in both microcosms of indigenous microorganisms and biostimulation. The highest degradation activity was found in the microcosm of the consortium together with biostimulation (49.5%) after 120 days of incubation in soil dosed with 10 g kg⁻¹ crude oil at 0 mM NaCl.

The degradation of ULO in microcosms of SC9 consortium with and without indigenous bacteria (SS+SCI, NSS+SCI) were 70.76% and 62.0%, respectively after 30 days of experiment (Figure 4). Bioaugmentation of SC9 consortium promoted ULO degradation activity even with indigenous bacteria. Similar observation has been reported for the use of bacterial consortium enhanced the biodegradation of hexadecane from soil and oily sludge from refinery in a slurry batch (Nozari, Samaei, & Dehghani, 2014). The aerobic biodegradation of complex petroleum products requires a mixed bacterial consortium with a variety of metabolic capacities to broad range of hydrocarbon compounds (Olajire & Essien, 2014). Many recent reports showed success in removal of a wide range of hydrocarbon compounds from soils using bacterial consortium (Adams, Fufeyin, Okoro, & Ehinomen, 2015; Patowary, Patowary, Kalita, & Deka, 2016). Fitri, Ni'matzahroh, and Surtiningsih (2016) reported the bioremediation of oil sludge contaminated soil using a different type of nitrogen source and the consortium of bacteria with composting method. The addition of NPK, *Azotobacter* and bacteria consortium were increase in the total number of bacteria with the value of 14.24 log CFUg⁻¹ soil and the percentage of degradation of 77.8% in 6 weeks of incubation. Abiotic loss of ULO (SS) due to photo-oxidation, volatilization and evaporation was also reported (Perfumo, Banat, Marchant, & Vezzulli, 2007).

In addition, pH of the all sterile soil treatment were relatively constant at around 6.90 throughout the experiment while pH of the all non-sterile soil dropped to around 5.52-5.63. The result of abiotic treatment (SS) showed no microorganisms. All non-sterile soil slurry treatments had greater initial microbial populations and also exhibited the increase number of microorganisms when determined from 0, 7 and 30 days of cultivation (Figure 5).

best treatment (NSS+SCI+CB) from weight loss of ULO was selected for GC-MS analysis. It was found that aliphatic compounds of C₁₆-C₂₁, benzene, xylene and naphthalene were the major compounds of the hydrocarbon extracted from ULO-contaminated soil slurry at the beginning (Figure 6a). At the end of the 30 days study, treatment of NSS+SCI+CB showed completely degradation of C₁₇-C₂₁, benzene, xylene and naphthalene. Some amount of C₁₆ were also degraded when compared with day 0, only C₁₆ was found as a major peak after 30 days of cultivation (Figure 6b). Fangru (2017) investigated alkane degradation of *Alcanivorax* sp. H34 non-growing cells in sterilization natural seawater. The results showed that the

optimal growth conditions of H34 were 30°C, initial pH 7.0, 3 g l⁻¹ nitrogen and 3 g l⁻¹ phosphorus. This strain can degrade moderate carbon chain of C₁₀-C₂₄. Paraffinic components average degradation rates and total degradation rate of H34 were 41.6 and 45.5%, respectively.

SC9 consortium together with crude biosurfactant has potential for apply in oil-contaminated soil, sediment and sludge. SC9 consortium and crude biosurfactant can enhance the biodegradation rate of other *ex-situ* techniques such as land-farming, biopile and composting. SC9 consortium would have a benefit to accelerate the indigenous bacteria to achieve in degradation of oil-contamination soil because it may have a variety of metabolic capacity to degrade a wide range of hydrocarbon compounds and may not take time to adapt after oil exposure.

3.7 Characterization of SC9 consortium

Profiles of PCR-DGGE of the SC9 consortium are summarized in Figure 7. Nine DNA bands were detected from the SC9 consortium and each band was labeled. Three strains could have function in ULO degradation were *Paenibacillus* sp., *Acinetobacter* sp. and *Stenotrophomonas maltophilia*. These strains were also found during enrichment cultures of ULO-contaminated soil from Songkhla Province (Meeboon, Kaewsuwan, Leigh, & Maneerat, 2016). These three strains have been reported to degrade aliphatic and aromatic hydrocarbons (Daane *et al.*, 2002; Jung, Noh, & Park, 2011; Tebyanian, Hassanshahian, & Kariminik, 2013), and to produce biosurfactants that enhance the solubility of aliphatic and aromatic hydrocarbons (Gudiña *et al.*, 2015; Singh *et al.*, 2015; Wong, Zhao, & Zheng, 2010). The nucleotide sequence was appeared in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB686241-AB686250.

4. Conclusions

This work shows the capability of SC9 consortium to degrade ULO in a soil slurry system. By evaluating all of the variables simultaneously, the best experimental conditions could be determined. Under the optimized conditions, SC9 consortium was able to degrade ULO about 63.3% after seven days of operation. The supplementation with SC9 consortium or crude biosurfactant produced by *A. calcoaceticus* subsp. *anitratus* SM7 all contributed to additional degradation ULO in non-sterile soil slurry condition (NSS+SCI+CB). The maximum degradation efficiency of 88.6% weight lost was achieved within 30 days. The SC9 consortium was also able to degrade aliphatic fraction and aromatic fraction present in ULO. SC9 consortium and biosurfactant derived from *A. calcoaceticus* subsp. *anitratus* SM7 have potential for apply in soil slurry bioremediation of ULO-contaminated soil.

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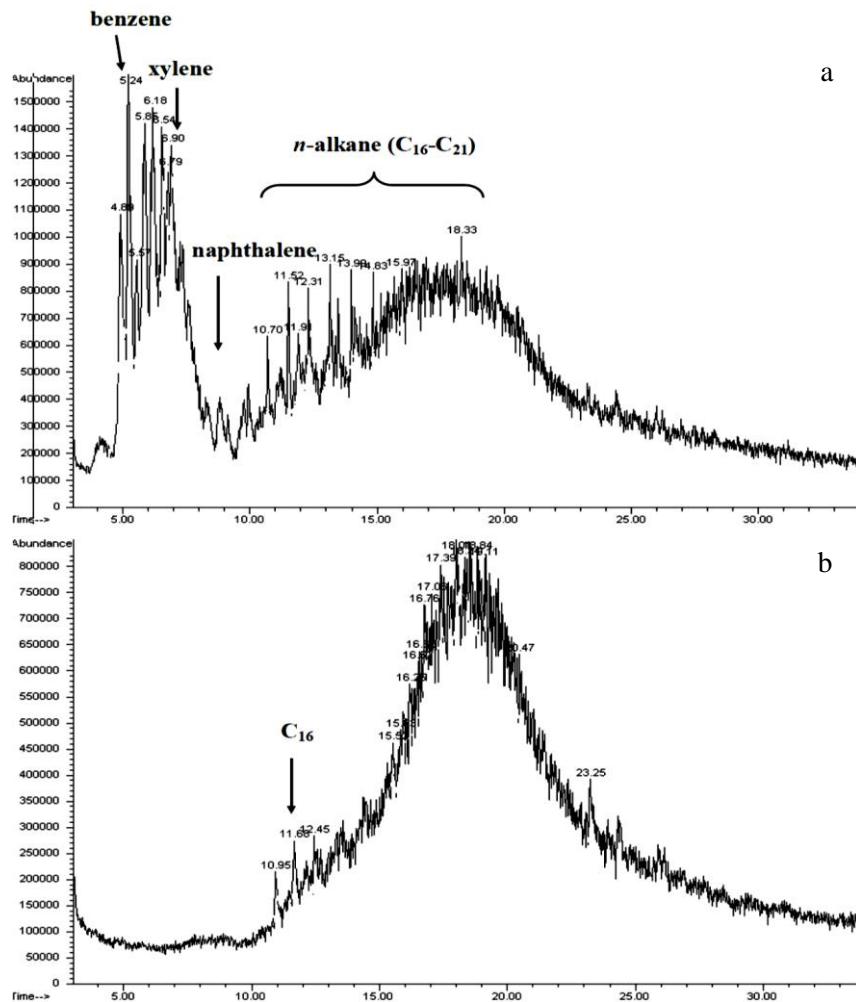


Figure 6. GC-MS chromatogram of hydrocarbon fraction from ULO contaminated soil slurry without sterilization supplemented with SC9 consortium and crude biosurfactant obtained from *A. calcoaceticus* subsp. *anitratus* SM7 (A= day 0, B= day 30). Time scale in minutes.

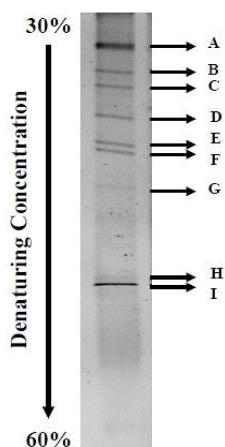


Figure 7. DGGE profiles of the 16S rDNA gene bands obtained from SC9 consortium.

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