

# Mineralization of Petroleum Contaminated Wastewater by Co-Culture of Petroleum-Degrading Bacterial Community and Biosurfactant-Producing Bacterium

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## ABSTRACT

Activity of a crude biosurfactant extracted from the culture fluid of *Serratia* sp. that was isolated from riverbed soil was shown to increase in proportion to the cultivation time, and was higher at pH 8 than at pH 7. *Serratia* sp. grew in the mineral-based medium with soybean oil but was not with kerosene-diesel. The petroleum-degrading bacteria—*Acinetobacter* sp., *Pseudomonas* sp., *Paracoccus* sp., and *Cupriavidus* sp.—were isolated from a specially designed enrichment culture. The efficiency of mineralization of wastewater contaminated with kerosene and diesel (WKD) by the petroleum-degrading bacterial community (PDBC) was enhanced significantly by addition of the crude biosurfactant. The efficiency of mineralization of the WKD was also about 2 times boosted by co-culture of *Serratia* sp. and PDBC. Bacterial community of *Serratia* sp. and PDBC co-cultivated in the WKD was maintained for at least 8 days according to the TGGE pattern of 16S rDNA obtained from the bacterial culture. In conclusion, the co-culture of *Serratia* sp. and PDBC is an applicable technique for the mineralization of wastewater contaminated with petroleum, which may substitute for chemical or biological surfactant.

**Keywords:** Biosurfactant, *Serratia* Sp. Petroleum-Degrading Bacteria, Mixed Culture, TGGE

## 1. Introduction

The petroleum hydrocarbons can be converted biologically or chemically to carbon dioxide, water, and inorganic compounds by dissimilatory metabolism of petroleum-degrading bacteria or combustion technique. Both biological and chemical conversion of petroleum hydrocarbons to inorganic compounds is defined as mineralization; however, the combustion technique can't be applied for mineralization of petroleum-contaminated wastewater and soil.

A variety of petroleum-derived compounds have contaminated soil and water in specific areas surrounding systems for the production, storage, distribution, and processing of petroleum by accidental spills and leakages. In particular, accidental petroleum leakages from underground storage tanks causes significant pollution of waters and soils [1]. For the successful intrinsic and engineered bioremediation of soil or water contaminated with

petroleum, the complex relationship existing among pollutants and microorganisms involved in contaminant degradation must be understood [2]. Light non-aqueous-phase liquids composed of petroleum hydrocarbon floating on water surface coagulate as the result of water fluctuations [3,4]. This phenomenon may effect the inhibition of hydrocarbon uptake by microorganisms or induce the adsorption of microorganisms onto oil drops. Theoretically, microorganisms within petroleum drops larger than bacterial size can lose their biological activity due to damage to the membrane. The emulsification process of utilizable carbon sources may constitute the rate-limiting step in the microbial degradation of petroleum hydrocarbon pollutants contaminating water.

Biosurfactants are unique amphipathic molecules that are metabolically generated by a variety of oil-utilizing microorganisms; they have been explored for possible use in a broad range of industrial and bioremediation applications [5-7]. Certain biosurfactants generated by

*Bacillus* species induce low interfacial tensions between the hydrocarbon and the aqueous phases required for the mobilization of petroleum hydrocarbons [8,9]. Several groups of biosurfactants have been identified as prerequisites for the formation of the fruiting body of *Bacillus subtilis* and the biofilm produced by *Pseudomonas aeruginosa* [10,11]. Nonaqueous-phase liquids, including nonpolar hydrocarbons, chlorinated solvents or manmade organic compounds, may be retained as relatively immobile and discontinuous globules [12-14]. The solubility or miscibility of the non-aqueous-phase liquids can be enhanced by chemically synthesized or biologically produced surfactants [15,16].

Four species of petroleum-degrading bacteria—*Pseudomonas* sp., *Cupriavidus* sp., *Paracoccus* sp., and *Acinetobacter* sp.—were isolated from an enrichment culture saturated with hydrocarbon vapors. Vapor of a hydrocarbon mixture was sparged into the enrichment culture medium in order to increase the probability of contact among hydrocarbon molecules and bacterial cells in aqueous phase. However, the sparging of petroleum or nonaqueous hydrocarbons into the bacterial culture is not an appropriate technique for application to treatment system for petroleum-contaminated wastewater.

In this study, a biosurfactant-producing bacterium isolated from soil and PDBC (petroleum-degrading bacterial community) isolated from an enrichment culture system were employed to improve the efficiency of mineralization of petroleum hydrocarbons-contaminated wastewater. The effect of a crude biosurfactant and a co-culture of the biosurfactant-producing bacterium and the PDBC on bacterial mineralization of WKD (wastewater contaminated with kerosene and diesel) was estimated and compared based on the TOC (total organic carbons) variation. The physiological stability of the biosurfactant-producing bacterium and the PDBC during co-cultivated in the WKD was analyzed using the TGGE technique.

## 2. Materials and Methods

### 2.1. Isolation of Microorganisms

Biosurfactant-producing bacterium was isolated from riverbed soil using a mineral-based soybean oil medium composed of 3 g/L of ammonium sulfate, 1 g/L of potassium phosphate monobasic, 3 g/L of potassium phosphate dibasic, 5 g/L of soybean oil, and 2 ml/L of trace mineral stock solution. The trace mineral stock solution was composed of 0.01 g/L of  $MnSO_4$ , 0.01 g/L of  $MgSO_4$ , 0.01 g/L of  $CaCl_2$ , 0.002 g/L of  $NiCl_2$ , 0.002 g/L of  $CoCl_2$ , 0.002 g/L of  $ZnSO_4$ , 0.002 g/L of  $Al_2[SO_4]_3$ , 0.001 g/L of  $CuSO_4$ , 0.002 g/L of  $MoCl_2$ , and 10 mM EDTA [17]. Ten grams of river bed soil was put in 100 ml of mineral-based soy bean oil medium and incubated

at 30°C and 150 rpm in a shaking incubator for 7 days. The bacterial culture grown in the mineral-based soybean oil medium was serially diluted up to  $10^8$  times with the fresh medium by 10-folded dilution method and then cultivated in same condition for 5 days. Maximally diluted medium in which bacteria were grown was serially diluted several times again. Finally, the purity of bacterial culture grown in the maximally diluted medium was estimated by TGGE technique.

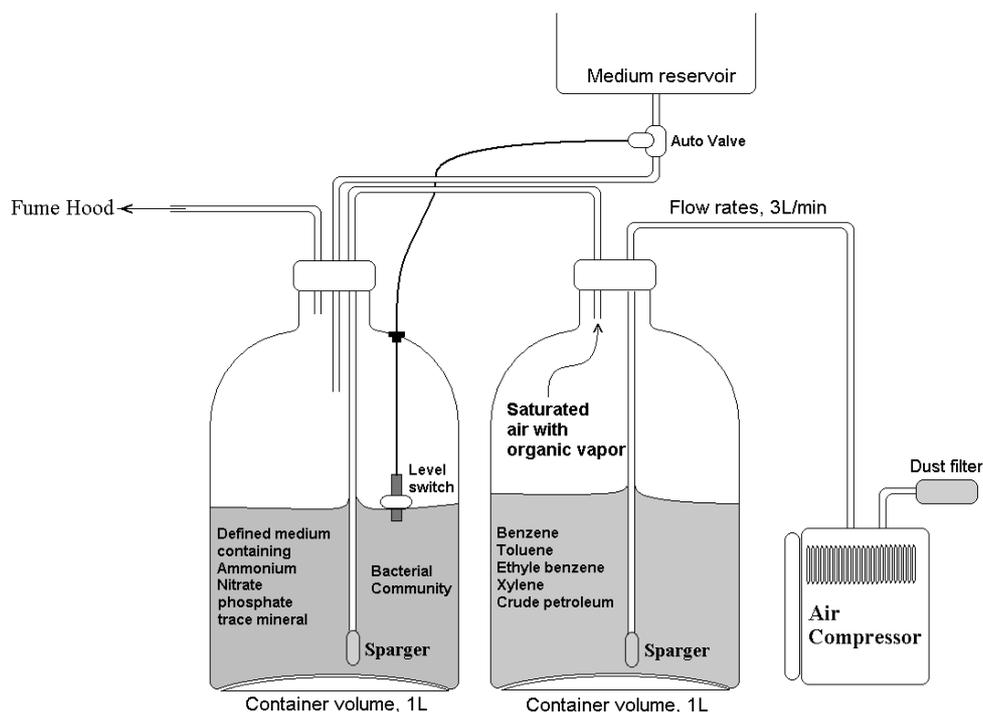
Petroleum-degrading bacteria were isolated from a bacterial culture enriched with petroleum, which was grown using a variety of hydrocarbons according to the method previously developed by Lee *et al.* [18]. A mixture of volatile hydrocarbons was flowed into the soil mixture suspension with air flow (3 L/min) by evaporation, as shown in **Figure 1**. One hundred  $\mu$ l of the bacterial culture enriched for more than 6 months was spread onto agar plates containing a mineral-based medium without organic compounds. The agar plates were placed into glass desiccators containing kerosene and diesel in the bottom. Air in the desiccator may be saturated naturally with the petroleum vapor evaporated from kerosene and diesel, by which the bacterial cells spread on the agar plates may contact with petroleum molecules and absorbed those. Desiccator cap was opened a time a day for 5 min to supply fresh air. Colonies emerged on the mineral-based agar medium were transferred to a mineral-based broth medium containing 5 g/L of kerosene and diesel, which was shaken vigorously at 250 rpm to induce dispersion of petroleum molecules into aqueous medium and incubated at 30°C.

### 2.2. Identification of Microorganisms

Chromosomal DNA was directly extracted from the bacterial isolates. 16S ribosomal DNA was amplified via direct PCR using the chromosomal DNA template and 16S-rDNA specific universal primers as follows: forward 5'-GAGTTGGATCCTGGCTCA G-3' and reverse 5'-AAGGAGGGGATCCAGCC-3'. The PCR reaction mixture (50  $\mu$ l) consisted of 2.5U of Taq polymerase, 250  $\mu$ M of each dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 100 ng template, 50 pM primer, and 1.5 mM  $MgCl_2$ . Amplification was conducted for 30 cycles of the following: 1 min at 95°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C using a PCR machine (T Gradient model, Biometra, German). Bacterial identity was determined on the basis of 16S-rDNA sequence homology, via the GenBank database system.

### 2.3. Temperature Gradient Gel Electrophoresis (TGGE)

TGGE technique is useful to effectively separate the



**Figure 1. Schematic structure of bioreactor for the enrichment of a bacterial community capable of degrading petroleum hydrocarbons. Various organic vapors can be transferred via air flow through the pipeline from the compressor to bacterial cultures. A one-tenth concentration of fresh medium balanced with evaporated volume was automatically refilled [19].**

variable region of 16S-rDNA by difference of temperature-dependent denaturation between AT and GC pair. The 16S-rDNA amplified from chromosomal DNA was employed as a template for the preparation of the TGGE sample (16S-rDNA variable region). A variable region of 16S-rDNA was amplified using a forward primer (eubacteria, V3 region) 341f 5'-CCTACGGGAGGCA-GC-AG-3' and reverse primer (universal, V3 region) 518r 5'-ATTACCGCGGCTGCTGG-3'. A GC clamp (5'-CGCCCCGCCGCGCGCGGGCGGGGCGGGG GCACGGGGGGCCTACGGGAGG-CAGCAG-3') was attached to the 5'-end of the GC341f primer [19]. The procedures for PCR and DNA sequencing were identical to the conditions used for 16S-rDNA amplification, with the exception of an annealing temperature of 53°C. The TGGE system (Bio-Rad, Dcode™, Universal Mutation Detection System, USA) was operated in accordance with the manufacturer's specifications. Aliquots (45 ml) of the PCR products were electrophoresed in gels containing 8% acrylamide, 8 M urea, and 20% formamide with a 1.5 x TAE buffer system at a constant voltage of 100 V for 12.5 hr and then at 40 V for 0.5 hr, applying a thermal gradient of 39°C to 52°C. Prior to electrophoresis, the gel was equilibrated to the temperature gradient for 30 min to 45 min.

#### 2.4. Amplification and Identification of TGGE Band

DNA was separately extracted from each TGGE band and purified using a DNA gel purification kit (Accuprep, Bioneer, Korea). The purified DNA was then amplified with the same primers and procedures used for TGGE sample preparation, in which the GC clamp was not attached to the forward primer. The species-specific identity of the amplified variable 16S-rDNA was determined based on sequence homology, according to the GenBank database system.

#### 2.5. Biosurfactant Activity Assay

Ten µl of *n*-decane containing Sudan III (0.001%, w/v) was dropped into distilled water (surface diameter, 90 mm) in a petri dish, resulting in the formation of an oil film. The surfactant can cause the oil film to spread and form a ring on the surface of the water. Cell-free culture fluid of *Serratia* sp., *Cupriavidus* sp., *Pseudomonas* sp., *Paracoccus* sp., and *Acinetobacter* sp. was dropped onto the oil film to evaluate surfactant production by the petroleum-degrading bacteria.

#### 2.6. Separation of Biosurfactant from Culture Fluid

Cell-free culture fluid was obtained by 30 min of cen-

trifugation at 5000  $\times g$  and 4°C. Five hundred ml of a chloroform-methanol (1:1) mixture was mixed with 5000 ml of the cell-free culture fluid in a separation funnel and then shaken for 100 strokes at 20°C for 120 min. The solvent phase was separated from the cell-free culture fluid and then the solvent was evaporated via N<sub>2</sub>-flushing. Finally, a viscous liquid remained, and was employed as a crude biosurfactant for the petroleum mineralization tests.

### 2.7. Effect of Biosurfactant on Mineralization of WKD

Unpurified domestic sewage was obtained from a man-hole of a pipeline flowing in an aerobic treatment reactor in a terminal disposal plant of sewage (Jungrang plant, Seoul, Korea). Two hundred ml of the sewage was prepared in a 500ml-medium bottle (reactor), to which 20 g/L of kerosene-diesel mixture was added to prepare WKD. The initial pH of the WKD was adjusted to 8 using ammonium hydroxide. PDBC that was previously cultivated in the mineral-based kerosene-diesel medium and harvested by centrifugation at 5000  $\times g$  and 4°C for 30 min was used as an inoculum. Each 10 g/L of the crude biosurfactant and PDBC based on wet weight was inoculated into the prepared WKD. No biosurfactant was added to but PDBC was inoculated into the WKD for the control test. The bacterial reactor was cultivated at 30°C in a 200 rpm of shaking incubator. All reactants employed in the bacterial petroleum mineralization test were prepared in triplicate. The mineralization activity of the petroleum-degrading bacteria was determined on the basis of the TOC variation.

### 2.8. Effect of Co-Culture on Mineralization of WKD

*Serratia* sp. that was previously cultivated in the mineral-based soybean oil medium and harvested by centrifugation at 5000  $\times g$  and 4°C for 30 min was used as an inoculum. Each 10 g/L of the harvested *Serratia* sp. and WKD based on wet weight was inoculated into the WKD of which initial pH was adjusted to 8 using ammonium hydroxide. No *Serratia* sp. but PDBC was inoculated into the WKD for the control test. The bacterial reactor was cultivated at 30°C in a 200 rpm of shaking incubator. All reactants employed in the bacterial petroleum mineralization test were prepared in triplicate. The mineralization activity of the petroleum-degrading bacteria was determined on the basis of the TOC variation.

### 2.9. TOC Measurement

The TOC was evaluated in accordance with the method organized by the HACH Company (Loveland, Colo,

USA). All procedures for the measurement of TOC were conducted in accordance with the instructions provided in the HACH manual (US Patent 6,368,870). Samples were diluted appropriately with distilled water within the range of detection. All chemicals used for TOC measurement were purchased from HACH. TOC was measured directly with a programmed spectrophotometer for automatic calculation (HACH model, DR/2500).

## 3. Results

### 3.1. Bacterial Identity

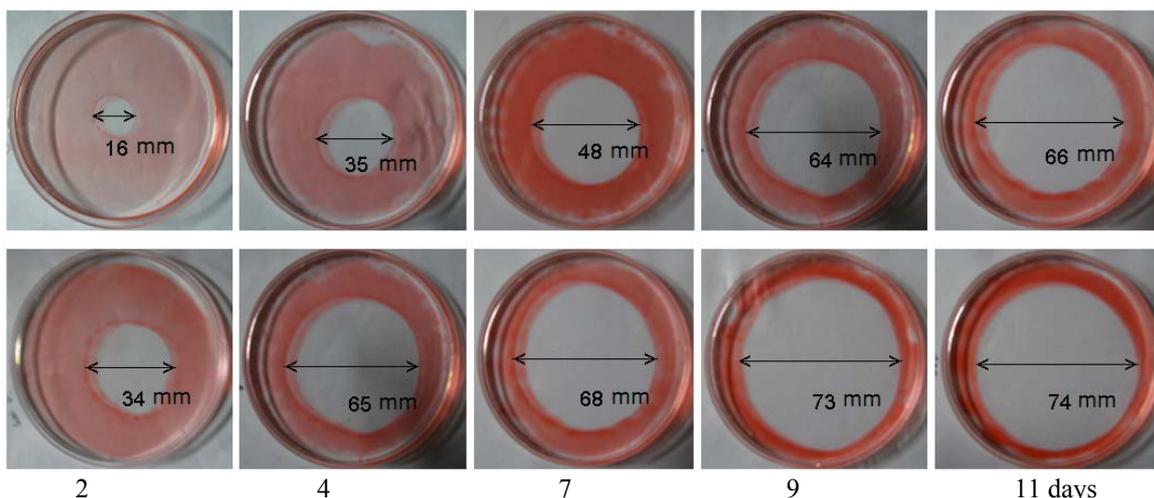
The 16S-rDNA variable region amplified with genomic DNA extracted from bacterial culture selected with mineral-based soybean oil medium was separated as a single band in TGGE (data not shown), which is the sign that the selected bacterial culture is pure. The purely isolated bacterium was identified based on 16S-rDNA sequence homology and registered in the GenBank database system, from which the following accession number was obtained: FJ971961, designating *Serratia* sp. SK090424. The petroleum-degrading bacteria were previously registered [18].

### 3.2. Biosurfactant Activity

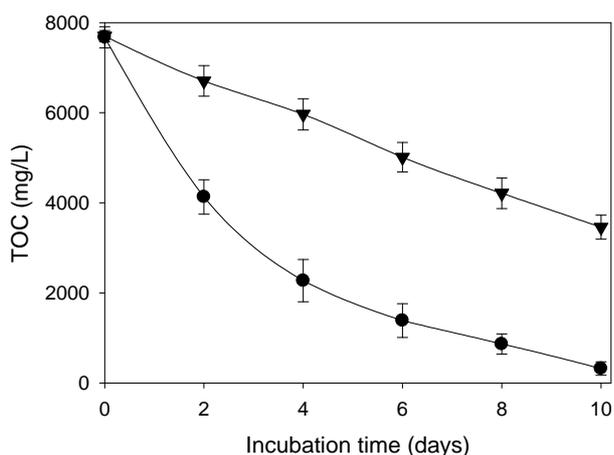
Biosurfactant production by *Serratia* sp. was proportional to cultivation time and was significantly higher at pH 8 than at pH 7, as shown in **Figure 2**. The relatively higher biosurfactant activity at pH 8 may be caused by the higher emulsification effect of alkaline condition for soybean oil. The emulsified oil may be absorbed and catabolized more actively by bacterial cells than the droplet oil.

### 3.3. Effects of Biosurfactant on Mineralization of Petroleum

Efficiency of mineralization of PDBC was about 2 times higher with biosurfactant than without as shown in **Figure 3**. The emulsification activity of PDBC for kerosene and diesel is presumed to be lower than *Serratia* sp. based on the enrichment process. In the process for enrichment or isolation, *Serratia* sp. was cultivated with soybean oil droplet but PDBC was cultivated exclusively with petroleum vapor that may be dispersed freely into bacterial culture. The nonaqueous kerosene and diesel droplet may be dispersed limitedly into bacterial cultures under condition without biosurfactant, and this condition permits the limited growth of PDBC, as well as the limited absorption of kerosene-diesel. The biosurfactant was quite effective in facilitating the catabolism of petroleum hydrocarbons by specific PDBC.



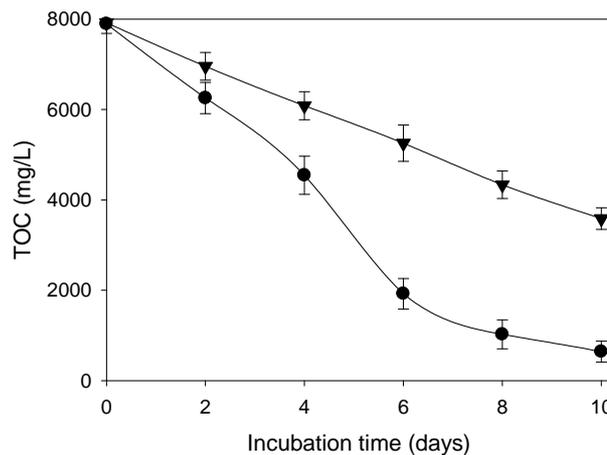
**Figure 2.** Oil spreading activity (diameter, mm) of biosurfactant produced by *Serratia* sp. SK090424 cultivated in the mineral-based soybean medium for 2, 4, 7, 9, and 11 days at pH 7 (upper) and 8 (lower).



**Figure 3.** Mineralization of WKD by PDBC in the condition with (●) or without the biosurfactant (▼).

### 3.4. Effect of Co-Culture of *Serratia* sp. and PDBC

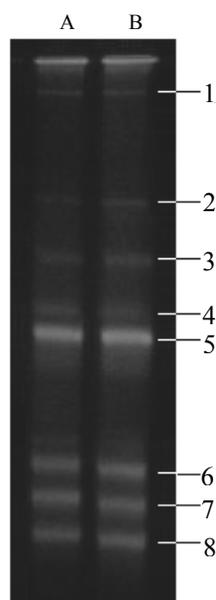
*Serratia* sp. was not grown when cultivated with petroleum vapor evaporated from kerosene and diesel; however, the efficiency of mineralization of WKD was increased by co-culture of *Serratia* sp. and PDBC, as shown in **Figure 4**. At the initial reaction time from 0 to 4 days, the mineralization by the co-culture was relatively lower than that by the addition of the biosurfactant; however, the efficiency was recovered at the later reaction time between 6 to 10 days. This result implies that some metabolites generated from kerosene and diesel hydrocarbons by PDBC metabolism may constitute useful nutrient for the growth and biosurfactant production of *Serratia* sp.



**Figure 4.** Mineralization of WKD by co-culture of *Serratia* sp. SK090424 and PDBC (●) or PDBC (▼).

### 3.5. Bacterial Community Variation during WKD Treatment

Eight of distinguishable DNA bands were observed on TGGE for the petroleum-degrading bacteria and the biosurfactant-producing bacteria cultivated in the WKD, as shown in **Figure 5**. In the TGGE pattern obtained on the 2<sup>nd</sup> and 8<sup>th</sup> day of incubation time, the DNA band for *Serratia* sp. was maintained. This result implies that *Serratia* sp. incapable of catabolizing the petroleum hydrocarbons may grow on some of the metabolites produced by the PDBC. Five of the eight partial 16S-rDNAs extracted from the TGGE bands were identified as the five bacterial species that had been initially inoculated into the AWKD, but the others were identified as uncultured bacteria and *Pseudomonas* sp., which may have



**Figure 5. Diversity of the bacterial community cultivated in the WKD at 2<sup>nd</sup> day (A) and 8<sup>th</sup> day (B) of incubation time. *Serratia* sp. and PDBC were inoculated into the WKD at initial time. The numbered DNA band was identified on the basis of sequence homology as follows: 1. uncultured bacterium; 2. *Pseudomonas* sp.; 3. *Paracoccus* sp.; 4. *Pseudomonas* sp.; 5. *Acinetobacter* sp.; 6. *Serratia* sp.; 7. *Cupriavidus* sp.; 8. *Pseudomonas* sp.**

originated from the unpurified sewage.

#### 4. Discussion

The biological mineralization of petroleum hydrocarbons may be one of the solutions for the remediation of petroleum-contaminated environments [20]; however, the low water miscibility or solubility of the petroleum hydrocarbons limits their availability to specific microorganisms capable of catabolizing petroleum hydrocarbons [21]. The biodegradation efficiency of the petroleum hydrocarbons may be increased in proportion to their solubility. Chemically synthesized or biologically generated surfactants have been employed in order to increase the solubility of the petroleum hydrocarbons and, by extension, to augment the efficiency of mineralization of the hydrocarbons [22,23]. The synthesized surfactants are relatively toxic for microorganisms and not particularly biodegradable, but the biosurfactants are non-toxic and biodegradable [24]. Both of the surfactants may prove problematic for application to oil-contaminated beaches, soils, or bioreactors based on the toxicity of synthetic surfactants to microorganisms and the microorganism-induced degradation of biosurfactant [25-27]. The toxicity of the synthetic surfactant may inhibit the

ability of the bacterial community to mineralize hydrocarbons, and the biodegradability of the biosurfactant may also induce an abrogation of its activity. The concentration of the surfactant in the reactant or in contaminated environments must be maintained at above-minimum levels for the successful emulsification of petroleum hydrocarbons, as both of the surfactants are effective to a limited extent for low-concentration application to contaminated sites [24]. This is the limiting factor in the application of both synthetic surfactants and biosurfactants to the bioreactor for the treatment of petroleum-contaminated wastewater. The ideal reaction conditions for the biosurfactant-mediated petroleum mineralization may be accomplished by the co-culture of biosurfactant-producing bacterium and petroleum-degrading bacteria, as the petroleum-degrading bacteria don't produce actively biosurfactant, but all of the biosurfactant-producing bacteria do not consistently degrade petroleum hydrocarbons [28,29]. The data shown in **Figure 3** demonstrate that the PDBC depends on the biosurfactant for the effective mineralization of petroleum hydrocarbons. The data shown in **Figure 4** demonstrate that the biosurfactant-producing bacterium, *Serratia* sp., depend upon the growth of PDBC in the WKD. *Serratia* sp. was shown to be unable to grow in the presence of petroleum hydrocarbons, but did grow and generate biosurfactant when co-cultured with the PDBC during growth in WKD. We conducted no tests to identify which bacterial strain of the PDBC produces metabolites for the growth of *Serratia* sp., but we may assume that the PDBC—*Pseudomonas* sp., *Cupriavidus* sp., *Paracoccus* sp., and *Acinetobacter* sp.—can generate a variety of metabolites, and some of these metabolites may function as substrates for the growth of *Serratia* sp.; additionally, certain of these metabolites may function as a substrate for biosurfactant production.

#### 5. Conclusions

A co-culture of biosurfactant-producing bacteria and PDBC may function as an effective substitute for synthetic surfactants or biosurfactants, as the limiting factors of the synthetic surfactant and biosurfactant may be supplemented by the biosurfactant continuously generated by growing cells of the biosurfactant-producing bacteria. The relationship between the biosurfactant-producing bacteria and PDBC may fall short of a true symbiosis, but is clearly sufficient for the synergetic mineralization of petroleum hydrocarbons. The co-culture technique tested in this study may be applicable to treatment systems for petroleum- or xenobiotics-contaminated wastewater based on the synergetic growth of the biosurfactant-producing bacteria and PDBC and the practical monitoring techniques of specific bacterial community.

Practically, various techniques that are real time PCR, DNA chip, and FISH (fluorescence *in situ* hybridization) have been employed to monitor the bacterial communities growing in bioreactors and natural ecosystems.

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