Effects of Rhamnolipid Biosurfactant on *Novosphingobium sp.* Bacteria for Enhanced Phenanthrene and Pyrene Degradation

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Abstract

The objective of this study was to investigate effects of rhamnolipid biosurfactant on *Novosphingobium sp.* by using phenanthrene and pyrene as carbon sources. Toxicology test and bacterial growth in presence and absence of rhamnolipid biosurfactant were determined by measuring OD_{600}. Results showed that rhamnolipid biosurfactant was nontoxic to bacteria growth. In addition, *Novosphingobium sp.* was able to use phenanthrene and pyrene as sole carbon and energy sources without biosurfactant at the maximum OD_{600} of 0.269 and 0.248 for 48 h as 0.1 initial. Addition of rhamnolipid biosurfactant concentration at 10 and 20 CMC was very useful to improve bacteria growth. The OD_{600} reached a maximum value of 0.704 and 0.871 for phenanthrene and 6.46 and 8.26 for pyrene at 24 h respectively. This reason was due to rhamnolipid can be used as sole energy and carbon sources for this strain. Addition of rhamnolipid biosurfactant to *Novosphingobium sp.* showed non adverse effect on bacterial growth and increased *Novosphingobium sp.* development for enhancing phenanthrene and pyrene biodegradation in the aqueous phase.

Keywords: *Rhamnolipid biosurfactant, phenanthrene, pyrene, biodegradation*

1. Introduction

Concern of petroleum hydrocarbons contamination in environment have gained much attention due to their content were reported as harmful organic compound (Pei et al.,2010). Polycyclic aromatic hydrocarbons (PAHs) were reported as having complex structure; hydrophobic and insoluble in water so that they are often persistent in the environment. In addition, PAHs have toxic, mutagenic and carcinogenic effect on microorganisms and humans (Rodrigues et al.,2013; Mori et al., 2015). Phenanthene and pyrene are group of PAHs priority pollutants listed by the US Environmental Protection Agency (USEPA). Generally, phenanthrene and pyrene can be removed by many mechanisms such as physicochemical and biological methods (Bautista et al.,2009; Janbandhu & Fulekar, 2011). Biodegradation methods have been used to monitor and enhance pollutant removal from contaminated sites by using natural microorganism. PAHs degradation efficiency depends on environmental condition, number and type of microorganisms including nature and the chemical structure of PAHs. *Novosphingobium sp.* is the one of the microorganisms that are present in natural petroleum contaminated sites which have ability to degrade PAHs. Several studies had succeeded in reducing PAHs contamination in soil using many bacteria species such as *Sphingobium, Pseudomonas, Arthrobacter species* and so on (Fu et al., 2014; Janbandhu & Fulekar, 2011; Rostami & Juhasz, 2013). However, some properties of PAHs such as low water solubility and dissolution rate are limits of biodegradation processes. Biosurfactants are surface-active compounds molecules which can be produced by wide variety of microorganisms. Rhamnolipid biosurfactant was categorized as anionic surfactant produced from *Pseudomonas aeruginosa* bacteria. Not only biosurfactants can reduce surface tension, interfacial tension of immiscible fluids; they can also increase solubility of PAHs (Zhao, Selvam, & Wong, 2011). Furthermore, many reports revealed that rhamnolipid biosurfactants have potential to enhance solubility and increase biodegradation of PAHs with *Flauobateriurn sp. Q14*, *P. Mycobacterium gilvum VM552, Bacillus subtilis BUM* and *P. aeruginosa P-CG3* (Congiu et al., 2015; Sun et al.,2008; Zhao et al., 2011). However, biodegradation of PAHs using *Novosphingbium sp.* with rhamnolipid biosurfactant are very limited.

2. Objective

The objective of this study was to investigate effects of rhamnolipid biosurfactant to phenanthrene and pyrene degrades bacteria in liquid medium.

3. Materials and methods

3.1 Microorganism

Novosphingobium sp. was selected to use as phenanthrene and pyrene degrading bacteria in this work. The bacterial seed was received from Department of Microbiology, Faculty of Science, Burapha University.

3.2 Biosurfactant

The anionic rhamnolipid biosurfactant (Jeneil Biosurfactant Co., LLC, USA) was produced by *Pseudomonas aeruginosa* from a mixture of 50% w/v monorhamnolipid and 50% w/v dirhamnolipid as shown in Figure 1 and Table 1. The critical micelle concentration of rhamnolipid from our previous work was 0.3 mM in deionized water and 1 mM NaCl as electrolyte (Keomany & Asnachinda, 2015).

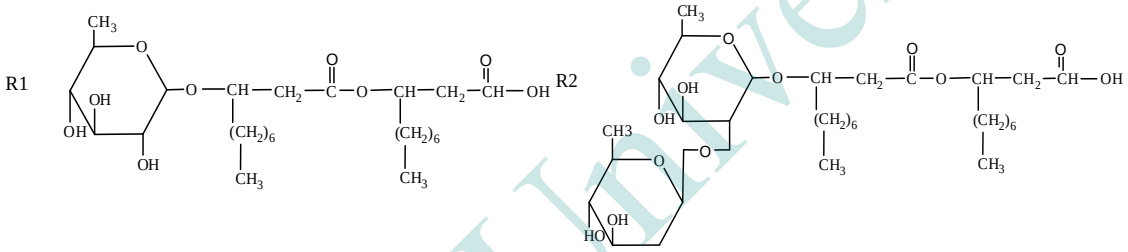

Figure 1 Structure of rhamnolipids, monorhamnolipid (R1) and dirhamnolipid (R2).

Table 1 Summarized properties of biosurfactants used in this study.

Surfactant: rhamnolipid	Average MW.	% Active	Molecular formula
Monorhamnolipids	504	16.2	$C_{26}H_{28}O_9$
Dirhamnolipid	650	16.2	$C_{32}H_{58}O_{13}$

3.3 Chemical

Phenanthrene and pyrene (both 98%, Arcos Chemical Company, Belgium) were selected to use in this study. One liter of carbon free mineral medium (CFMM) at pH 7.5 consisted of 3 g NH_4NO_3, 0.8 g KH_2PO_4, 5.5 g $Na_2HPO_4.12H_2O$, 0.1g $MgSO_4.7H_2O$, 0.05g $FeCl_3.6H_2O$, 0.05g $CaCl_2.2H_2O$, 15 g agar was used for culturing bacteria.

3.4 Microorganism growth

Stock of *Novosphingobium sp.* pure culture from -80°C was streaked onto carbon free mineral medium (CFMM agar) saturated with 20,000 ppm of phenanthrene vapor. Culture samples were then incubated at 30°C for 5 days for observation of bacterial growth.

3.5 Toxicity of surfactant on bacterial growth.

In this part, toxicity of rhamnolipid biosurfactant on the survived *Novosphingobium sp.* was investigated. One colony of *Novosphingobium sp.* was applied to CFMM as control and supplement to biosurfactant concentrations at 0.5, 1, 5, 10, and 20 folds of CMC in 250 mL flasks containing 50 mL of liquid CFMM. After that, samples were shaken at 200 rpm for 7 days. Samples were collected once daily from day 0 to day 7 to observe bacterial growth by the optical density measurement at 600 nm (OD_{600}) (Mesbaiah et al., 2014; Sarakhun, 2005).

3.6 Effects of rhamnolipid biosurfactant on microbial growth in phenanthrene and pyrene

Colony of *Novosphingobium sp.* was transferred to 250 ml flasks containing 50 ml of carbon free mineral medium (CFMM). Samples were then shaken at 200 rpm for 24 h and centrifuged at 6,000 rpm for 15 min with controlled temperature of 4°C to harvest the cell. Then, cells were washed twice in 0.85% NaCl and suspended in the same solution. The cells concentration was adjusted to optical density of 0.1 at OD_{600} nm by using 0.85 NaCl and resting cells were done by shaking at 200 rpm for 24 h. After that, 0.1 ml suspension was added in a test tube containing 5 ml CFMM at pH 7.5. A 100 ppm of each phenanthrene and pyrene were also added to the same test tube and biosurfactant concentrations from toxicology test samples were further incubated by shaking at 200 rpm, 30°C for 7 days. All samples were collected as triplicates from 0-7 days to examine effects of rhamnolipid biosurfactant to phenanthrene and pyrene degrading bacteria.

4. Results and discussion

4.1 Toxicology assay

Effects of rhamnolipid biosurfactant on the bacterial growth were evaluated in different concentrations of rhamnolipid at 0.5, 1, 5, 10 and 20 folds of CMC. Samples were incubated for 7 days and the results are presented in Figure 2. Results demonstrated that *Novosphingobium sp.* increased with increasing rhamnolipid biosurfactant concentrations from 0.5 to 20 folds of CMC. These results may elucidate that rhamnolipid biosurfactant was utilized as a sole carbon and energy source of this strain. Findings were also implied that rhamnolipid biosurfactant was not toxic to *Novosphingobium sp.* and promoted bacterial growth subjected to surfactant concentrations. Results in Table 2 demonstrated growth of *Novosphingobium sp.* measured by OD_{600} with addition of rhamnolipid biosurfactant as carbon source in various concentrations. *Novosphingobium sp.* in controlled condition and 0.5 CMC of rhamnolipid biosurfactant slightly increased from 0-6 days (0.033-0.076) and 0-7 days (0.33-0.760). In addition, *Novosphingobium sp.* was increased from 0-4 days (0.044-0.078) for concentration at CMC. While, *Novosphingobium sp.* supplement with rhamnolipid biosurfactant concentrations at 5, 10 and 20 folds of CMC increased dramatically in one day (0.170; 0.317 and 0.612 respectively). These results indicated that rhamnolipid biosurfactant have positive effects on *Novosphingobium sp.* growth. Result from this experiment was in agreement with literatures which suggested that addition of surfactant into bacteria culture can enhance the growth of bacteria (Aryal & Liakopoulou-Kyriakides, 2013; Mesbaiah et al., 2014; Sun et al., 2008).

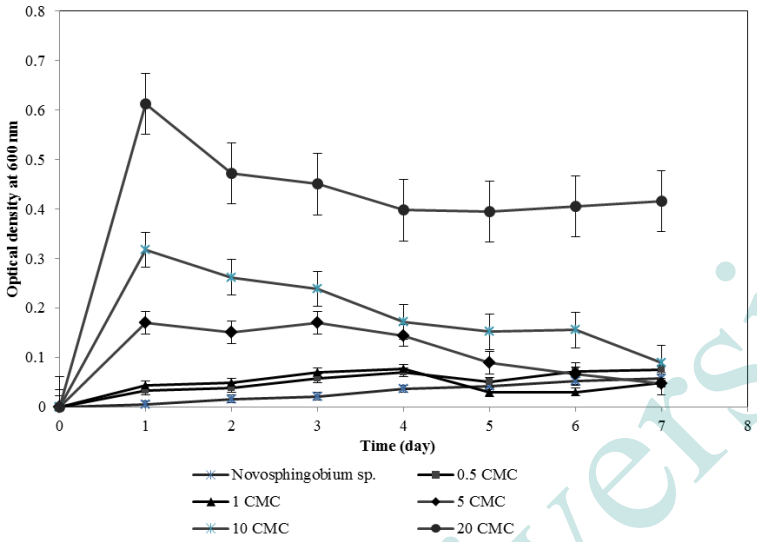

Figure 2 Relationship of optical density at 600 nm and incubation time of *Novosphingobium sp.* at different concentrations of rhamnolipid as 0.5, 1, 5, 10 and 20 folds of CMC.

Table 2 Summary data of rhamnolipid biosurfactant to *Novosphingobium sp.* growth after being incubated for 7 days.

Day	Optical density at 600 nm					
	Control condition (*Novosphingobium sp.* + CFMM)	Addition of rhamnolipid biosurfactant				
		0.5 CMC	1 CMC	5 CMC	10 CMC	20 CMC
1	0.005	0.033	0.044	0.170	0.317	0.612
2	0.016	0.038	0.048	0.150	0.262	0.472
3	0.023	0.057	0.070	0.170	0.239	0.450
4	0.039	0.070	0.078	0.144	0.172	0.398
5	0.045	0.050	0.030	0.090	0.152	0.396
6	0.061	0.071	0.030	0.066	0.155	0.405
7	0.060	0.076	0.049	0.049	0.090	0.415

Toxicology test was conducted for seven days to ensure that rhamnolipid biosurfactant have no adverse effects on bacteria. Results showed that rhamnolipid biosurfactant was not toxic to bacteria used to degrade phenanthrene and pyrene. In addition, bacterial growths were performed at the highest value on rhamnolipid concentrations of 10 and 20 folds of CMC. Thus, those concentrations of rhamnolipid were selected to use in the next experiment. The study of rhamnolipid biosurfactant for enhancing phenanthrene and pyrene biodegradation by free cell of *Novosphingobium sp.* was examined in 5 ml CFMM. Each CFMM cultures were supplemented with concentration of 100 mg/L of phenanthrene and pyrene and triplicated samples were taken at seven days.

4.2 Effects of rhamnolipid biosurfactant to microbial growth on phenanthrene and pyrene

The growth curves of the *Novosphingobium sp.* during phenanthrene and pyrene as control and addition of rhamnolipid biosurfactant concentration at 10 and 20 CMC after inoculation for seven days were shown in Figure 3 and 4. The bacterial growth with phenanthrene as control and addition of rhamnolipid biosurfactant as it carbon source is shown in the Figure 3. This figure indicated that the optical density of

Novosphingobium sp. was significantly increased from 0.1 initially up to 0.269 by addition of phenanthrene. On the other hand, rhamnolipid biosurfactant addition at concentrations of 10 and 20 CMC were increased to 0.704 and 0.871 after 24 h, respectively. Figure 4 showed bacteria growth in the pyrene and rhamnolipid biosurfactant additions. It was demonstrated that *Novosphingobium sp.* could grow in both conditions with and without rhamnolipid biosurfactant as well as in phenanthrene. The optical density of *Novosphingobium sp.* in the presence of rhamnolipid biosurfactant concentrations of 10 and 20 CMC with pyrene were increased to 6.46 and 8.26 for 24 h (initial optical density 0.1). While in the absence of rhamnolipid biosurfactant, the optical density showed only 0.248 for 48 h. This demonstrated that rhamnolipid biosurfactant was very useful to improve bacterial growth due to rhamnolipid biosurfactant can reduce interfacial tension and enhance solubilization of phenantheren and pyrene in aqueous phase, and can be utilized as sole carbon sources of this bacteria strain as well. However, bacterial growth will be high or less depending on biosurfactant concentration as well (Sun et al., 2008; Whang et al., 2008; Zhao et al., 2011).

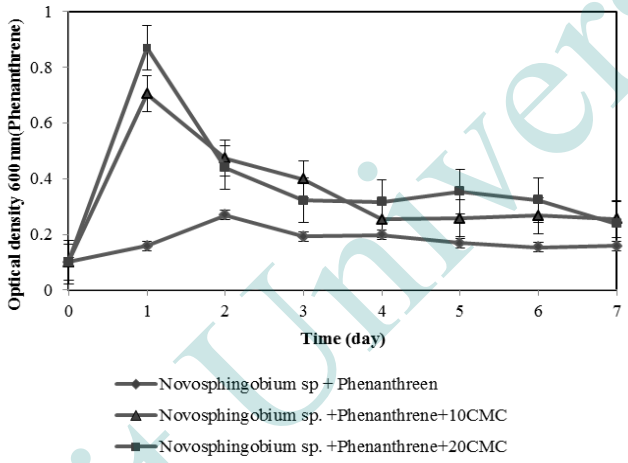

Figure 3 Relationship of optical density at 600 nm and incubation time of *Novosphingobium sp.* in presence of phenanthrene with different concentrations of rhamnolipid biosurfactant.

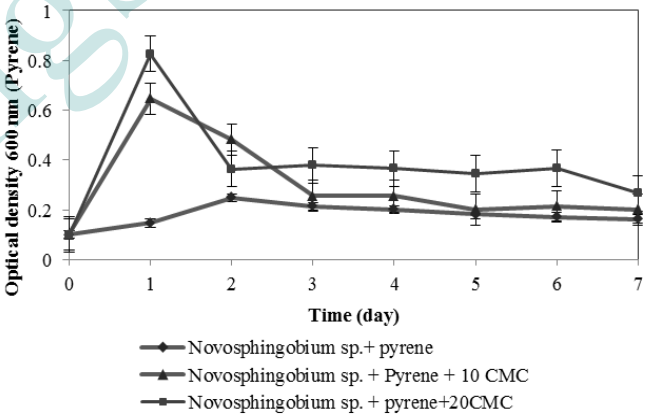

Figure 4 Relationship of optical density at 600 nm and incubation time of *Novosphingobium* sp. in presence of pyrene with different concentrations of rhamnolipid biosurfactant.

5. Conclusion

Results from these work demonstrated that rhamnolipid biosurfactant have no adverse effect on *Novosphingobium sp*. On the other hand, rhamnolipid was non-toxic and friendly to use as phenanthrene and pyrene degrading bacteria. Moreover, addition of rhamnolipid biosurfactant to *Novosphingobium sp.* increased bacterial growth and encouraged phenanthrene and pyrene degradation as sole carbon and energy sources in aqueous phase.

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