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Enhanced Biodegradation of Hydrocarbons in Soil by Microbial Biosurfactant, Sophorolipid

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Abstract Effectiveness of a microbial biosurfactant, sophorolipid, was evaluated in washing and biodegradation of model hydrocarbons and crude oil in soil. Thirty percent of 2-methylnaphthalene was effectively washed and solubilized with 10 g/L of sophorolipid with similar or higher efficiency than that of commercial surfactants. Addition of sophorolipid in soil increased biodegradation of model compounds: 2-methylnaphthalene (95% degradation in 2 days), hexadecane (97%, 6 days), and pristane (85%, 6 days). Also, effective biodegradation method of crude oil in soil was observed by the addition of sophorolipid, resulting in 80% biodegradation of saturates and 72% aromatics in 8 weeks. These results showed the potentials of the microbial biosurfactant, sophorolipid, as an effective surfactant for soil washing and as an in situ biodegradation enhancer.

Keywords Sophorolipid · Petroleum hydrocarbon · Bioremediation · Microbial surfactant

Introduction

Oil pollution causes a great environmental problem to terrestrial and marine ecosystems. Biodegradation of petroleum-contaminated soils requires effective and complex remediation techniques [1]. Major components of petroleum, hydrophobic organic compounds (HOCs), have low aqueous solubility and strong binding/sorption onto solids. Solubilization of HOCs increased bioavailability and thereby improved the biodegradation rate [2–4]. A common remediation process of contaminated sites is based on the extraction by organic solvents or surfactants. Unfortunately, many HOCs that are common groundwater pollutants cannot be efficiently extracted because of their low solubility in water and high interfacial tensions. Recently, surfactant-enhanced remediation techniques have been proposed for potential use in in situ soil remediation as soil flushing agents and enhancers for bioremediation [5–7].

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Surfactants are surface-active agents that have amphiphilic properties consisting of hydrophilic polar head moiety and a hydrophobic non-polar tail moiety. Surfactants can increase extraction of hydrophobic materials through micellar solubilization and mobilization of such materials through a reduction in their interfacial tension and increase the apparent solubility of HOCs in water [1, 5, 8–11]. Therefore, surfactants are useful for the biodegradation of low-solubility hydrocarbon-contaminated soil and enhancing the biodegradation rate of petroleum hydrocarbons in a contaminated soil. Surfactant solubilization and enhancement of biodegradation is significantly influenced by the size and structure of the hydrocarbon contaminant by physical/chemical effects of surfactant [1, 7, 10, 12–14].

The bioremediation abilities are completed naturally by microbial systems, accelerated through the use of suitable enhancers and represented as bioavailability [15–20].

Bioavailability may be the critical limiting factor controlling biodegradation rates for many organic compounds having low water solubility. Biodegradation rate depends on salinity, temperature, pH, heavy metals surfactants, nutrients, and presence of readily available carbon sources for assisting in the mineralization of some petroleum components into carbon dioxide and water [21–23]. Several nonionic surfactants and biosurfactants give us good insight into the biodegradation kinetics of HOCs due to their non-toxicity and enhancing the aqueous solubility [18, 24, 25]. More research is necessary to make the application of surfactants a standard tool in biological soil remediation [26].

Microbial surfactants are more effective and environmentally friendly than chemical synthetic surfactants. Sophorolipid, a microbial glycolipid, is produced from *Candida bombicola*. Cultivation techniques to increase the productivity have been developed using various methods, which increase its concentration up to 100–300 g/L and exhibits high productivity and non-toxicity. Sophorolipid can also be converted from various renewable and locally available resources, such as vegetable oil, fatty acid, animal fats, alkanols, and recycled products. This availability could make it one of the most promising alternatives to synthetic surfactants [27–29].

In this study, we investigated a potent microbial surfactant, sophorolipid, as a possible soil flushing agent and enhancer for bioremediation in comparison to synthetic surfactants for applications in bioremediation.

Materials and Methods

Materials

Surfactants Synthetic surfactants (Tween 20, 60, 80, 81 and Span 20, 80, 85) were purchased from Sigma-Aldrich Co. Ltd., and their chemical properties such as hydrophilic lipophilic balance (HLB) and molecular weight are described on Table 1. Series of Tween and Spans were used as high and low HLB surfactants, respectively.

Preparation of Sophorolipid *C. bombicola* ATCC 22214 was stored on YM slants at 4 °C. The production medium contained, per liter, 100 g of glucose, 5 g of yeast extract, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of NaCl, 0.7 g of peptone, and 10% (*w*/*v*) of corn oil. Five percent (*v*/*v*) of culture suspension was used as the inoculums for a 2.5-L jar fermenter (Kobiotech, Korea). Culture conditions were as follows: working volume, 1 L; temperature, 30 °C, pH 3.5; agitation, 550 rpm; aeration rate, 1 VVM; and culture time, 7 days. The culture broth was extracted three times with the same volume of ethyl acetate.

Table 1 Characteristics of surfactants for soil flushing and bioremediation.

Surfactant	Structure	Molecular weight (Da)	HLB
Tween 20	Polyoxyethylene sorbitan mono laurate	1,227.54	16.0
Tween 60	Polyoxyethylene sorbitan mono stearate	1,311.70	14.9
Tween 80	Polyoxyethylene sorbitan mono oleate	1,310.00	15.0
Tween 81	Polyoxyethylene sorbitan mono oleate	649.00	11.0
Span 20	Sorbitan monolaurate	346.46	8.6
Span 80	Sorbitan monooleate	428.61	4.0
Span 85	Sorbitan trioleate	957.51	1.0
Sophorolipid	Sophorose + fatty acid (C14–22)	550–800	4–8

To obtain crude sophorolipid, the ethyl acetate layer was evaporated by a rotary vacuum evaporator (Eyela, Japan) and residual oil was removed by extracting with *n*-hexane.

Contaminants Iranian light, a crude oil, was used as a contaminant which consisted of saturates (35±2%), aromatics (28±3%), and polars (37±2%). Representative HOCs were selected among aromatics and the linear/branched aliphatics. Selected compounds were 2-methylnaphthalene, *n*-hexadecane, and 2, 6, 10, 14-tetramethyl pentadecane (pristane) and were used for comparing the soil flushing effect and bioremediation as an artificial contaminants.

Soil Soil samples were collected randomly from a plant farming zone at Inha-University, Incheon, Korea. The sample depth was 20 cm. For experiments, samples were homogenized, air-dried, sieved between 0.6 and 1.7 mm through no. 12 and no. 30 sieves (U.S.A. standard testing sieve) and stored in polythene bags at 4 °C before use. Properties of soil sample were as follows: 1.23 g/cm^3 of bulk density, 2 g/cm^3 of particle density, 2.6% of moisture content, and 30.18% of saturated moisture content according to the hydrometer method [4].

Assays

Crude Oil The composition of crude oil was determined by thin layer chromatography/flame ionization detection (TLC/FID; IATROSCAN, Japan) and conditions were as follows: thin layer, Chromarod-S III; spotting volume, 1 μL; primary mobile phase, *n*-hexane; secondary mobile phase, *n*-hexane/toluene (1:4); H_2, 160 mL/min; air, 2.0 L/min; and scan speed, 30 s [30].

Hydrocarbons The quantification of HOCs was determined by gas chromatography equipped with FID (GC-14B, Shimadzu, Japan). Operating conditions of the GC were as follows: column, Silicon OV-101 (2 m SUS); initial temperature, 200 °C; final temperature, 240 °C; program rate, 5 °C/min; injection temp, 200 °C; detection temp, 240 °C.

Solubilization of Hydrocarbon by Surfactants in Aqueous Solution

Aqueous solubility experiments were performed in a 20-mL vial using 10 mL of various concentrations of sophorolipid and nonionic surfactants in the presence of excess amounts

of 2-methylnaphthalene. The vials were equilibrated for 48 h and 2 mL of samples was collected and extracted with 4 mL of *n*-hexane. The extracts were analyzed using gas chromatography. The molar solubility ratio (MSR) was expressed as the molar concentration of the hydrocarbon (2-methylnaphthalene) dissolved in the aqueous solution containing the sophorolipid and nonionic surfactants [2, 29].

Soil Flushing Effect of Surfactants on HOCs-Contaminated Soil

The rate and extent of flushing of the HOCs from the soil with surfactants were measured using batch experiments. The prepared soils used in this study were sterilized by autoclaving (121 °C and 15 psi) for 1 h. After autoclaving, for artificial adsorption of 2-methylnaphthalene into the soil, 2-methylnaphthalene (8% of soil, *w*/*w*) was dissolved in *n*-hexane solution and then poured onto the soil. The *n*-hexane was evaporated under a hood before the experiment and left for 24 h. The 30-mL vials contained 5 g of soil adsorbed with 2-methylnaphthalene and the surfactant solution (5–25 g/L, where the ratio of soil to solution was 1:5). The temperature and mixing rate during the experiment (12 h) were maintained at 25 °C and 200 rpm, respectively [7, 17]. After flushing, for removal of the soil, samples were centrifuged at 4,500 rpm for 20 min and the solubility of hydrocarbon in an aqueous solution was measured using gas chromatography.

Biodegradation of Artificial Contaminants and Crude Oil

Biodegradation experiments with artificial contaminants or crude oil were conducted at room temperature on a rotary shaker (200 rpm, Vision, Korea) with or without autoclaved soil and contained 0.20 g of glucose, 0.005 g of yeast extract, 0.50 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.10 g of $CaCl_2 \cdot 2H_2O$, 1.00 g of Na_2HPO_4, and 0.20 g of citric acid per liter of deionized water [6, 7, 10]. Biodegradation experiments were performed in 500-mL flasks. Each flask contained 50 g of contaminated soil which was contaminated by 0.6% of artificial contaminants (hydrocarbon mixture: *n*-hexadecane/pristine/2-methylnaphthalene= 1:1:1) or 0.8% of crude oil. Contaminants were dissolved in the *n*-hexane solution and then poured onto the soil. *n*-hexane was evaporated under a hood for 24 h. One hundred fifty milliliters of nutrient solution and 150 mL of surfactant solution were mixed with the contaminated soils. The flasks were incubated in an orbital shaker at room temperature, pH 7, and 200 rpm for 9 weeks. To determine the degree of biodegradation, samples (3-mL suspension) were withdrawn and centrifuged for 20 min at 4,500 rpm. Supernatant or soils were extracted with *n*-hexane, respectively. Extract was analyzed by GC/FID (artificial contaminant) or TLC/FID (crude oil). All experiments were carried out in duplicate.

Results and Discussions

Soil Flushing Effect of Surfactants

To determine the rate and extent of flushing of the HOCs from the soil with surfactants, molar solubility ratios of each surfactant against 2-methylnaphthalene were measured. The rate of partitioning of 2-methylnaphthalene to the aqueous phase was measured in sterile solution to ascertain whether the rate of utilization was determined by transfer of the chemical to the aqueous phase. Desorption in the soil slurry phase was conducted to assess the effect of different concentrations of sophorolipid. Tween 80/60/20 and Span20/80/85

were used to release 2-methylnaphthalene from the autoclaved solids. With surfactants present, the dissolution of 2-methylnaphthalene into the aqueous phase was dependent on the concentration and HLB of the surfactants (Fig. 1). The surfactants with a higher HLB (Tween 80, Tween 60, Tween 20) resulted in better solubility, indicating that the solubility of 2-methylnaphthalene is affected by the structure of the surfactants in the micelle. The MSR, which is the ratio of the number of moles of organic compounds dissolved per mole of surfactant added to the solution, was obtained from the solubility curve [6, 7]. In Fig. 1, the 2-methylnaphthalene solubility was plotted as a function of surfactant solution concentration. The 2-methylnaphthalene solubility was hyperbolically correlated to the surfactant concentration in the range of 0–25 g/L. The trend of profiles showed that the 2-methylnaphthalene solubility was restricted by the aqueous solubility of the surfactants. Tween 80 showed the highest molar solubility ratio against 2-methylnaphthalene among the tested nonionic surfactants. Surfactants of 8–15 of HLB were found to be suitable for soil flushing through high solubilization of HOCs (Fig. 1).

Sophorolipid has a lower aqueous solubility than the Tween series, but it showed a higher soil flushing efficiency than any of the other tested nonionic surfactants except Tween 80. Thus, effective factors for soil flushing may be not only HLB, cloud point of temperature, and macro-emulsion formation but also specific surfactant–organic interactions. Accordingly, structural varieties of sophorolipid could increase the specific surfactant–organic interactions and lead to increasing the aqueous solubility ratio of 2-methylnaphthalene and sophorolipid and could also increase the soil flushing efficiency through interactions of between contaminants and the sophorolipid.

Effect of Sophorolipid and Surfactants on the Biodegradation of Artificial Contaminants

A biodegradation experiment with artificial contaminants was conducted at room temperature on a rotary shaker with or without autoclaved nutrients. Biodegradation for each hydrocarbon was measured for 11 days. Most HOCs were degraded within 72 h (Fig. 2). Sophorolipid showed high biodegradation rate in most of the cases. In the case of *n*-hexadecane, both sophorolipid and Tween 80 showed that reduced contaminant concentration below 50 ppm in the soil during 2 days. Nutrient and moisture were essential factors for biodegradation of HOCs. Nutrients and water could potentially enhance the bioavailability of aliphatic hydrocarbons by microbial population growth of the soil. In

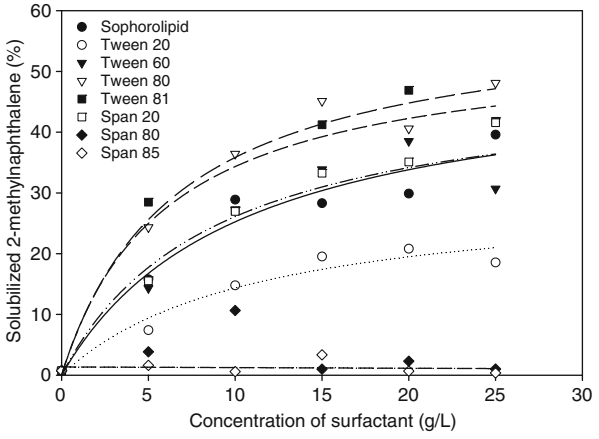

Fig. 1 Effects of surfactant concentration on the washing of 2-methylnaphthalene (washing by water only, 0.7433±0.0058%). Series of Tween were used as high HLB surfactants, whereas Spans were used as low HLB surfactants

addition, Tween 80 showed similar efficiency with sophorolipid and the highest efficiency among the nonionic surfactants (Fig. 2a). In the case of the aromatic compound, 2-methylnaphthalene, the biodegradation rates were slower than *n*-hexadecane, but the rank of efficacies was same with the aliphatic compound (Fig. 2b). In the case of the branched aliphatic compound, pristane, biodegradation results showed that sophorolipid was the best enhancing (Fig. 2c).

Biodegradation of Crude-Oil-Contaminated Soil Using Sophorolipid

Iranian light was used as a crude oil contaminant. To determine the optimum concentration of sophorolipid for biodegradation on crude-oil-contaminated soil, 0–3,000 ppm of

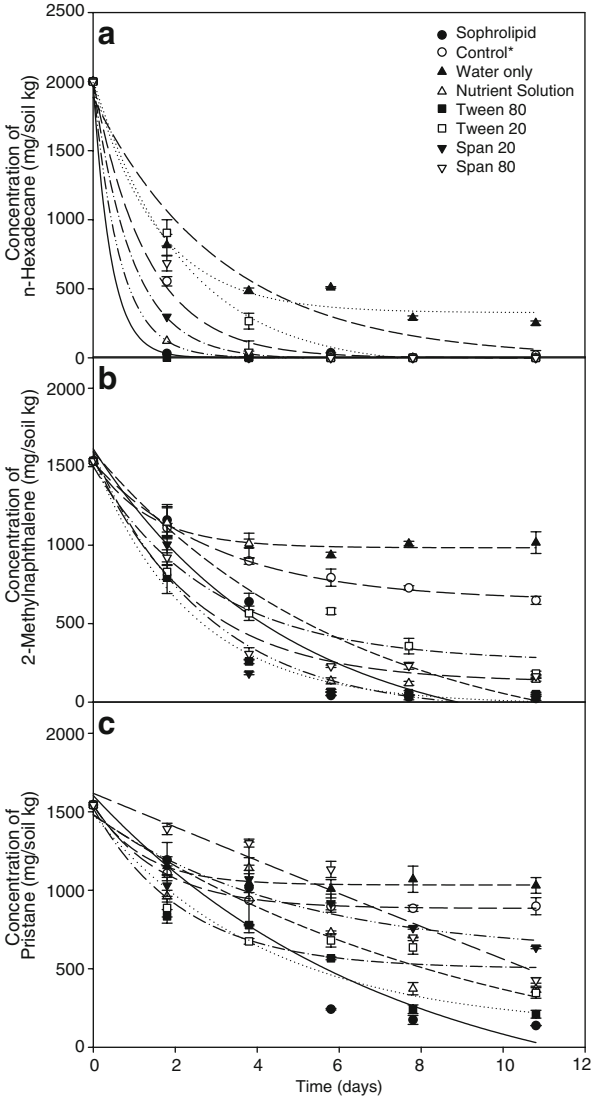

Fig. 2 Effects of various surfactants on the biodegradation of model contaminants in soil; *n*-hexadecane (**a**), 2-methylnaphthalene (**b**), and pristane (**c**). *Control; sterilized soil with sophorolipid addition. Surfactants (sophrolipid, Tween 80/20 and Span 20/80) were mixed with nutrient solution

sophorolipid was employed as an enhancer of remediation. Polars were not degraded at any conditions as abiotics, but the aromatics and the saturated aliphatics were prominently degraded by sophorolipid treatment (Table 2). Concentration of saturated carbons was dramatically reduced at 100 ppm of sophorolipid as in the aromatics. At the optimum condition, 2,000 mg/kg of sophorolipid showed 84.8±0.35% biodegradation rate for saturates. The biodegradation rate of crude oil was enhanced by surfactants for all hydrocarbon species except polars (Table 3). Hence, experimental sets were established with/without nutrients and sterility. Removal patterns of the hydrocarbons were similar among nonionic surfactants and sophorolipid. Surfactants (nonionic surfactant + sophorolipid) enhanced the biodegradation capacity of crude oil except for polars. Polars were not degraded in any of cases. The abiotic negative control (sterilized soil and no treatment) showed little physical losses over the experimental period. In contrast, the biodegradation rate of water and nutrient treatment for saturates and aromatics showed about 60%. These results showed that the biodegradation capacity was increased by not only the addition of biosurfactant/nonionic surfactants but also the addition of water and nutrients. It means that surfactants could enhance the biodegradation of HOCs in crude oils through the production of micelle encapsulation of HOCs, therefore increasing the bioavailability to microbial consortia. The major cause of biodegradation was microbial mineralization by growth of microbes. The set of nutrient-treated samples showed higher degradation capacity than the set of sophorolipid-treated samples without nutrients. Therefore, sophorolipid and tested nonionic surfactants were found to be suitable for use as an enhancer for biodegradation of microbes through the result of sophorolipid treatment without nutrients.

The effect of sophorolipid at various concentration of crude oil was tested. A 2,000-mg/kg quantity of sophorolipid could biodegrade 8,000 ppm of crude oil. In a sample containing 12,000 ppm of crude oil, the biodegradation capacities of saturates and aromatics were approximately decreased by 68% and 54%, respectively. The 15–20% decrease means that biodegradation was limited by the emulsifying capacity of sophorolipid and the toxicity of the crude oil (Table 4). To determine the effect of water contents in soil, moisture contents of soil were measured as the percent of water holding capacity using a hydrometer method. Also, 2,000 ppm of sophorolipid was used as an enhancer for biodegradation with nutrient treatment, and various concentrations of water were mixed with contaminated soils. Samples containing 50% of water content showed the highest biodegradation capacity on aromatics and saturates. Above the optimum condition, the biodegradation capacity was significantly reduced. Perhaps, the bioavailability of aerobic microbes was inhibited by

Table 2 Effects of sophorolipid concentration on biodegradation of crude oil components in soil.

Concentration of sophorolipid (mg/soil kg)	Degraded saturates (%)[a]	Degraded aromatics (%)	Degraded polars (%)
0	0.0±5.71	0.0±10.71	0.0±8.11
100	70.1±3.73	70.4±8.64	0.4±7.33
300	71.9±2.66	68.0±1.24	0.0±5.54
500	71.4±3.17	68.7±4.11	0.6±3.05
1,000	81.3±6.11	75.6±2.90	0.7±8.08
2,000	85.2±1.01	82.2±3.62	0.8±4.69
3,000	77.6±3.90	70.7±1.14	0.3±2.34

[a] Percent degraded after 8 weeks of biodegradation experiment with nutrients (8,000 ppm crude oil)

Table 3 Biodegradation of crude oil (Iranian light; including saturates, aromatics, and polar) with addition of various surfactants.

Surfactants	Degraded saturates[a] (%)	Degraded aromatics (%)	Degraded polars (%)
Sophorolipid[a]	80.7±1.14	71.7±1.24	4.8±3.07
Tween 80[a]	76.5±1.55	69.1±0.57	0.1±1.83
Tween 60[a]	72.9±1.18	70.1±1.38	0.2±2.13
Tween 20[a]	77.3±0.22	72.3±0.52	2.7±1.05
Span 20[a]	69.0±3.35	67.6±1.26	3.7±3.33
Span 80[a]	76.8±1.43	70.9±0.82	4.8±0.80
Span 85[a]	79.0±1.85	71.1±0.15	5.7±0.60
Water[a]	66.0±1.88	61.4±0.58	5.4±2.83
Water only	20.6±0.88	42.4±0.98	2.3±3.04
Sophorolipid only	32.6±1.04	41.8±0.81	5.0±1.92
No treatment[b]	0.0±2.19	29.6±1.62	0.0±2.0

[a] With nutrients; percent degraded after 8 weeks of biodegradation experiment (8,000 ppm crude oil)

[b] Using the sterilized soil

restrictions originated from the paste slurry that prohibited air transfer. But in the case of polars, the biodegradation capacity was increased as the water content increased. This result showed that the solubilization of polars might be increased by water content and its bioavailability could be increased by water solubilization as the increase of flushing efficiency (Table 5).

The biodegradation condition of crude oil was optimized as 2,000 ppm of sophorolipid with 8,000 ppm of crude oil contaminated soil with nutrients and 50% of water content during 8 weeks. Figure 3 shows time-dependent biodegradation rates at optimum condition. Iranian light, crude oil, was used as a contaminant which consisted of saturates (35±2%), aromatics (28±3%), and polars (37±2%). Saturates and aromatics were employed as model contaminants for measuring biodegradation rates. At this time, initial concentrations of saturates and aromatics were 2,800±160 and 2,240±240 ppm, respectively. Water was more effective in aromatics, and sophorolipid could enhance the biodegradation rate in both aromatics and saturates. Sophorolipid enhanced the biodegradation rate about 15% ($p<0.01$, Fig. 3a) on saturates and 12% ($p<0.05$, Fig. 3b) on aromatics in comparison with sets

Table 4 Effect of crude oil concentration on biodegradation of crude oil in soil.

Concentration of crude oil[a] (mg/kg)	Degraded saturates (%)[b]	Degraded aromatics (%)	Degraded polars (%)
4,000	82.7±3.21	87.3±7.51	12.0±1.41
8,000	81.0±1.73	70.0±2.00	5.0±2.83
12,000	68.0±6.93	54.3±5.13	2.0±11.2
16,000	73.7±2.31	54.0±5.29	11.0±8.48
20,000	69.7±3.21	48.3±7.57	18.0±2.83

[a] Iranian light

[b] Percent degraded after 8 weeks of biodegradation experiment. Nutrients and 2,000 ppm of sophorolipid was used as an enhancer for biodegradation

Table 5 Effect of water content in soil on biodegradation.

Moisture contents[a] (%)	Degraded saturates (%)[b]	Degraded aromatics (%)	Degraded polars (%)
0	0.04±6.27	28.43±5.07	0
30	69.32±17.69	59.00±3.74	9.27±11.02
50	78.53±4.90	70.86±1.53	3.70±6.17
70	42.89±12.31	61.51±6.59	4.00±7.41
90	7.04±4.91	38.71±2.99	8.77±4.11
100	0.08±3.29	25.33±5.00	19.50±2.23

[a] Moisture contents of soil were measured as the percent of water holding capacity by using a hydrometer method. 2,000 ppm of sophorolipid was used as an enhancer for biodegradation with nutrient treatment

[b] Percent degraded after 8 weeks of biodegradation experiment (8,000 ppm crude oil)

of nutrient-treated samples. The final concentrations of saturates and aromatics in the cases of sophorolipid treatments were 432±162 and 618±119 ppm, respectively. As the result, sophorolipid showed high efficiency for enhancing the biodegradation on saturates and aromatics. Therefore, sophorolipid could be applied to and were suitable for soil bioremediation of refined oils, which have no polars, such as diesel, gasoline, and kerosene, as an enhancer. In addition, adequate conditions (water and nutrients) of soil would be helpful to increase the bioavailability of microbial consortia.

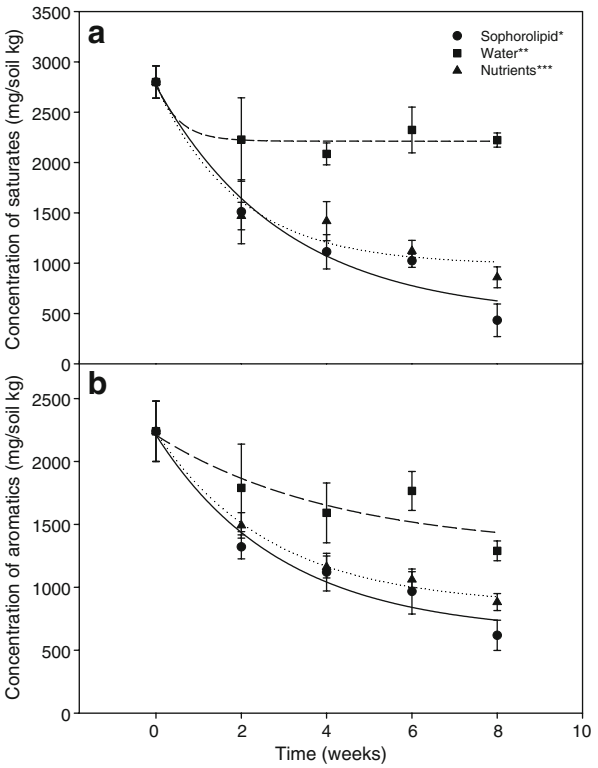

Fig. 3 Time course biodegradation of crude oil with surfactant addition. **a** Saturate, **b** aromatics. *Sophorolipid + nutrients; **water only; ***nutrients only

Conclusions

A microbial surfactant (biosurfactant), sophorolipid, was investigated for its potential to enhance bioavailability and, hence, the biodegradation of crude oil. Sophorolipid used in this study was extracted from culture supernatants after growth of *C. bombicola* ATCC 22214 on glucose and corn oil as substrates. Sophorolipid showed higher soil flushing efficiency than any other tested nonionic surfactants except Tween 80. In addition, sophorolipid was the best enhancing agent for biodegradation in artificial contaminants (including *n*-hexadecane, 2-methylnaphthalene, and pristine). Sophorolipid could be also applied to and were suitable for soil bioremediation of refined oils, which have no polars, such as diesel, gasoline, and kerosene. These results indicate that sophorolipid may have potential for facilitating the bioremediation of sites contaminated with hydrocarbons having limited water solubility and increasing the bioavailability of microbial consortia for biodegradation.

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