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Culture of Bacterium *Bacillus subtilis* as Degradation Agent for Sea Water Remediation Contaminated by Petroleum

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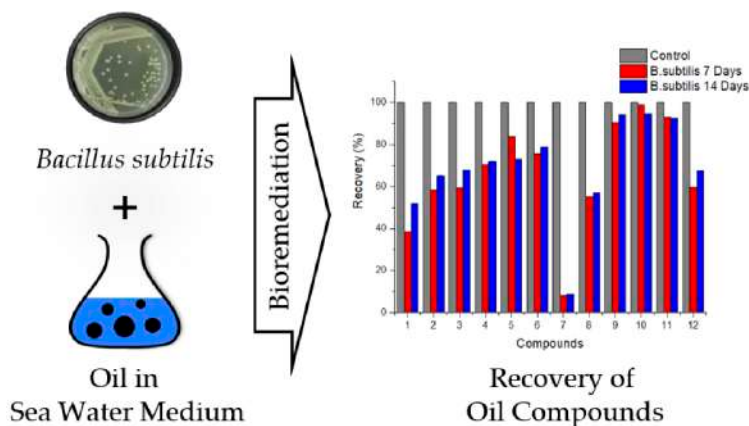
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Abstract

Crude oil spills pose a serious threat to the marine environment. This is due to crude oil, in large part, is composed of aromatic, aliphatic, and alicyclic hydrocarbons which are toxic, carcinogenic, and mutagenic to the marine life. However, the degradation of crude oil spills with bacteria in simulated seawater media is rarely reported. In this study, oil spill in the seawater, especially petroleum, had been successfully degraded by bacterium culture *Bacillus subtilis* in simulated seawater under 7 and 14 days incubation. Simulated seawater had synthesized based on ASTM D1141-98 for Standard Practice for the Preparation of Substitute Ocean Water. The petroleum recovery was analyzed using Gas chromatography-mass spectrometry. In this research, the optimum recovery value of crude oil degradation by the bacterial culture obtained by octadecadienoic acid compound gave a percentage recovery of 8.20% and 8.87% after 7 and 14 days of incubation, respectively. This result indicated that the *B. subtilis* culture has the ability to degrade crude oil spill in simulated sea water.

Biodegradation of crude oil in simulated seawater by *Bacillus subtilis* in 7 days of incubation time. Almost all compounds in the crude oil decreased substantially. The higher biodegradation was obtained in octadecadienoic acid compound. This result indicated that *B. subtilis* can be used as the alternative solution for bioremediation of sea water contaminated by petroleum.



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Keywords: *Bacillus subtilis*, Biodegradation, Bioremediation, Oil, Petroleum, Simulated sea water

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Introduction

Crude oil spills pose a serious threat to the marine environment. It occurs mainly due to leakage during the process of their transportation, refining, exploration, production, and storage. It has a bad impact on marine ecosystems because crude oil, in large part, is composed of aromatic, aliphatic, and alicyclic hydrocarbons which are toxic, carcinogenic, and mutagenic to the marine life [1,2]. Besides, some components of crude oil can accumulate (bioaccumulation) in susceptible organisms and over the long term the contaminant compounds will move from one organism to another through the food chain [3,4].

The handling of crude oil-contaminated environmental conditions can be done physically, chemically, and biologically. Physical and chemical remediation are short-term, but still giving bad secondary effects to the environment, and especially physical methods can only remove 10-15% of pollutants from the marine media [5,6]. Bioremediation is a pollution treatment method that is considered more environmental-friendly and effective than other methods to degrade crude oil spills because it uses microorganisms as a degrading agent [7–10]. The success of bioremediation in treating hydrocarbon-contaminated environments is dependent on the accessibility of pollutants to degrading organisms, the biodegradability of the pollutants and the optimization of biological activity [11]. The bioavailability of crude oil hydrocarbon compounds is limited due to their natural hydrophobic properties. The presence of a surfactant can increase oil mobility so that the degradation rate will increase. Biosurfactants which are naturally generated by bacteria can reduce the surface tension of oil so that the crude oil will be more soluble. The more soluble crude oils can increase the accessibility of crude oils to the degrading bacteria and help them to consume hydrocarbon compounds in crude oils [12].

Several genera of bacteria have been known as good hydrocarbon-degrading agents because they tolerate high concentration of hydrocarbons. Most of them are *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Arthobacter*, *Brevibacterium*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Xanthomonas* and *Bacillus* species [13,14]. *Bacillus* sp. is one of those bacteria that has been reported to have the capability to tolerate the high levels of oils because of their resistant endospores. This is evident from several studies which reported that the *Bacillus*

sp. is one of the dominant bacterial consortia that can be isolated from several polluted environments contaminated by crude oil spills. For example, research by Sorkhoh *et al.* [15] reported that 368 genera of *Bacillus* isolated from oil-polluted Kuwaiti desert and two strains were capable of degrading 80-89% of crude oil (5 g/L) within 5 days at high temperature (60 °C). Recently, Mulani *et al.* [12] have also reported that the genus *Bacillus* sp. which can grow efficiently on crude oil in coastal areas Vadinar, Gujarat, India, can degrade polyhydrocarbon cyclic (PHC) by 50% for 7 days. Meanwhile, the study by Sakthipriya *et al.* [16] showed that *Bacillus subtilis* strain bacteria had the capability to reduce the viscosity of wax crude oil by 60-80% for 10 days. Thus, microorganisms isolated from contaminated environments are generally the most active microorganisms for crude oil-degrading agents and can be used in bioremediation of environments contaminated by crude oil. However, the degradation of crude oil spills with *B. subtilis* bacterium in simulated seawater media is rarely reported. Therefore, this study aims to determine the biodegradation of crude oil in simulated seawater with exogenous bacterium *B. subtilis* NBRC 3009 (National Institut of Technology and Evaluation (NITE), Japan). Degradation of crude oil was analyzed and evaluated quantitatively by GCMS as well as identified the metabolites.

Experimental Section

Materials and Apparatus

The materials used are boric acid (H_3BO_3), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), potassium bromide (KBr), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), sodium sulfate (Na_2SO_4), strontium chloride ($SrCl_2$), sodium fluoride (NaF), acetone, aqua DM, *n*-hexane, dichloro diphenyl trichloroethane (DDT), potato dextrose broth (PDB), nutrient broth (NB), nutrient agar (NA), methanol, oxygen, and petroleum (Pusdiklat Migas, Cepu, Central Java).

Simulated Seawater Preparation

The manufacture of simulated seawater followed the procedure described in the "Standard Practice for the Preparation of Substitute Ocean Water" ASTM D1141-98. The dosage was made for 1 L of seawater, and the quantity of each ingredient was shown in Tabel 1.

Table 1. Tables Preparation composition of stock solutions.

Solution 1		Solution 2	
Salt	Mass (g)	Salt	Mass (g)
MgCl ₂ .6H ₂ O	13.8900	KCl	1.7375
CaCl ₂	1.4475	NaHCO ₃	0.5025
SrCl ₂ .6H ₂ O	0.0525	KBr	0.2500
		H ₃ BO ₃	0.0675
		NaF	0.0075

* Solutions I and II were dissolved in 25 mL of aqua DM

NaCl (24.534 g) and Na₂SO₄ (4.094 g) were dissolved in 1 L aqua DM. Gradually, solution I was added, subsequently followed by solution II, while stirring. The pH of the resulting simulated seawater was tested by pH meter giving a pH value of 8.36, which is in accordance with the pH of simulated seawater in the literature in the pH range of 7.5 - 8.4 [17,18].

Bacteria Preparation

The bacterium *B. subtilis* NBRC 3009 was purchased from Biological Resource Center (NBRC), National Institut of Technology and Evaluation (NITE), Japan. The *B. subtilis* stock was maintained on NA. The colony was inoculated into 10 mL NB in Falcon tube and incubated in the shaker at 180 rpm for 20 hours at 37 °C [19].

Biodegradation of Petroleum by *B. subtilis*

The pre-incubated bacteria were centrifuged for 10 minutes, NB liquid media was decanted and washed with 10 mL sterile water. Sterile water media were decanted and replaced with simulated seawater media of 10 mL in 100 mL Erlenmeyer flask. Further on, 50 µL of crude oil was added to each of the prepared cultures. Oxygen was subsequently added and incubated in static conditions at 30 °C for 7 and 14 days. As a negative control, crude oil was added in seawater medium without the addition of *B. subtilis*. The experiments were performed in triplicate [19].

Petroleum Recovery

The recovery was carried out by the addition of 50 µL of 5 mM DDT in DMSO (final concentration 0.25 µmol) as internal standard, followed by homogenization with 20 mL of methanol and washing with 5 mL of acetone. The culture was filtered with a 0.2 µm filter. After evaporation at 40

°C, the filtrate was extracted with *n*-hexane 3 times (total 300 mL). The extract was filtered with cotton and Na₂SO₄ placed on the funnel. The filter sample was evaporated to about 2 mL remaining, then 10 µL sample was injected and analyzed using gas chromatography-mass spectrometry (GC-MS) [19].

Crude oil recovery compounds were analyzed using Agilent Technologies 7890 GC System. The GC system was linked to a 30 m × 50 µm × 0.25 µm Agilent 19091S-433 column and an Agilent Technologies 5975C VL MSD Detector with. The oven temperature was programmed to start from 80 °C and held for 2 min and then the temperature was increased to 280 °C at 5 °C min⁻¹ and held for 15 min [19].

Results and Discussion

Analysis of Degradation Products (GC-MS)

Figure 1 showed the data of crude oil obtained from GC-MS analysis. The chromatogram showed the occurrence of crude oil degradation in the culture of 7 days and 14 days marked by the decreasing intensity of the peak area of the chromatogram from each compound when compared to the control sample. The greatest decrease in chromatogram intensity was demonstrated by the sample treated for 7 days and there was an increase in chromatogram intensity during the 14th treatment day. This might be due to decreasing ability of *B. subtilis* in degrading crude oil, as reported by Sakthipriya *et al.* [16], in which the degradation rate of wax crude oil with *B. subtilis* did not show any improvement after the 10th day.

In addition, an increase in the intensity of the compound on the 14th day occurred because shorter chain aliphatic hydrocarbons experience an accumulated amount from long-chain aliphatic hydrocarbon metabolites and short-chain aliphatic hydrocarbons that have not been degraded. Short-chain hydrocarbon compounds were degraded first during 7 days. It was then followed by the degradation of compounds with longer chains during the second half of 7 days to the 14th day giving result to an accumulation of the number of shorter chain hydrocarbon compounds on the 14 days and made the peak intensity of the compound rise.

The GC-MS data were then processed into a percent recovery comparison data of the control sample, 7 days treatment and 14 days treatment as shown in Figure 2. Percent recovery can be defined as the quantity of non-degradable compounds during

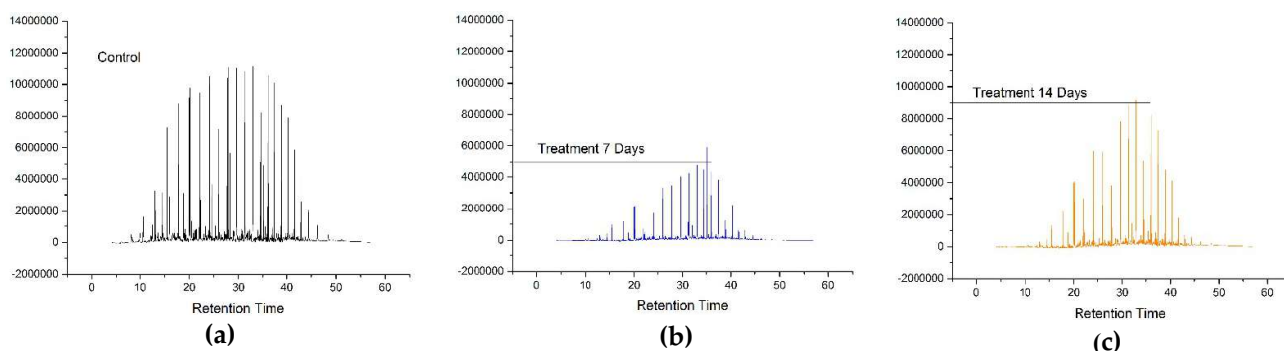


Figure 1. Chromatogram of crude oil recovery by *B. subtilis* for (a) control without bacteria, (b) treatment for 7 days, and (c) treatment for 14 days.

Table 2. Recovery result of metabolites compounds.

No.	Compounds	Recovery (%)		Degradation (%)	
		7 Days	14 Days	7 Days	14 Days
1	Hexadecane	38.38	52.09	61.62	47.91
2	Heptadecane	58.52	65.10	41.48	34.90
3	2,6-dimethyl-Heptadecane	59.39	67.90	40.61	32.10
4	Octadecane	70.67	71.87	29.33	28.13
5	2,6,10,14-tetramethyl-Hexadecane	83.95	72.81	16.05	27.19
6	Nonadecane	75.45	78.74	24.55	21.26
7	9,12-Octadecadienoic acid	8.20	8.87	91.80	91.13
8	11-Octadecenoic acid	55.07	57.05	44.93	42.95
9	Docosane	90.40	94.04	9.60	5.96
10	Hexacosane	98.97	94.47	1.03	5.53
11	Tricosane	92.82	92.41	7.18	7.59
12	Tetracosane	59.71	67.73	40.29	32.27

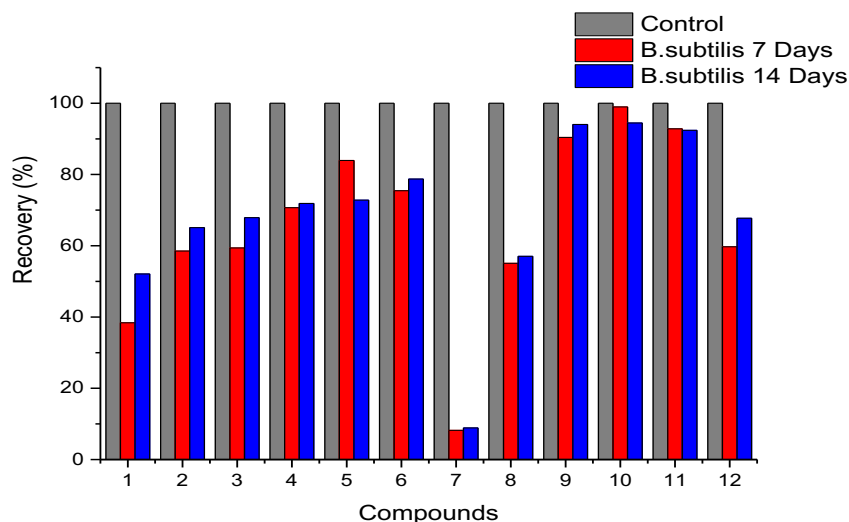


Figure 2. Petroleum recovery by *B. subtilis* bacteria.

the bioremediation process so that compound having a high percent recovery value means that the degradation of the compound was very low.

The types of compounds and percentage of recovery/degradation of each compound were shown in Table 2. Most of these compounds were

long-chain alkane compounds starting with hexadecane (C16) to C24 chain hydrocarbons. Furthermore, carboxylic acid (methyl ester) compounds may also be degraded by *B. subtilis* with a greater average percentage of degradation than long-chain alkane compounds.

The result showed that the optimum percentage recovery value for 7 and 14 days incubation exhibited by octadecadienoic acid, which were 8.20% and 8.87%, respectively. In other words, the compound can be degraded as much as 91.80% and 91.13%, respectively. The least degraded compound was found to be C26 long-chain alkane compounds (alkane with the longest degradable carbon chains) with a recovery percentage of 98.97% and 94.47%, or 1.03% and 5.53% degradation, for 7 and 14 days respectively.

The result of this study was supported by the previous studies. In the previous studies, the *B. subtilis* already reported having the capability to treat the crude oil spills. Das *et al.* [20] reported that *B. subtilis* DM-04 showed a significant reduction of Total Petroleum Hydrocarbon (TPH) in soil medium for 120 days incubation. In other study, Oyetibo *et al.* [21] reported that *B. subtilis* M16K and M19F showed excellent crude oil degradation up to 94% and 85% in Mineral Salt Medium (MSM) for 28 days incubation. It shows that *B. subtilis* can be a good biodegradation agent for crude oil remediation [21]. However, crude oil remediation by *B. subtilis* in simulated seawater medium has not been reported yet. In our previous report, biodegradation of crude oil under high salinity medium was carried out with *Ralstonia pickettii*. Octadecadienoic acid was also found in lower concentrations than control in crude oil biodegradation by *R. pickettii* under high salinity medium [19]. It means that some bacteria have great capabilities in biodegradation of octadecadienoic acid in crude oil remediation due to their capability to use long chain alkane such as octadecadienoic acid and hexadecanoic acid as the carbon source [22].

The degradation of a carboxylic acid by *B. subtilis* involves only the process of forming acetyl-CoA with β -oxidation, while long-chain alkane compounds need to be activated first with oxygenase enzyme, then oxidation reaction with O_2 reactant takes up until methyl terminal alkane became carboxylic acid group a degradation process followed by β -oxidation [23,24]. Therefore, carboxylic acid compounds are more easily degraded than long-chain alkane compounds [25]. In fact, the longer the hydrocarbon chains that these compounds possess, the more specific the enzyme which is required [26].

Besides through the degradation pathway of oxygenase enzyme, degradation with *B. subtilis* can also be done in a method of decreasing the surface tension of the insoluble component of the oil with the biosurfactant surfactin produced by *B. subtilis* [27].

The biosurfactant is an active molecular group of heterogeneous surfaces having areas with hydrophobic and hydrophilic properties to separate the interface between the fluid phases with different degrees of polarity, such as oil-water or air-water interfaces, thus reducing the surface or interfacial tension [28]. The lower surface or interfacial tension of petroleum can simplify the biodegradation process [28].

This study is still in the preliminary stage of research to establish the ability of *B. subtilis* to degrade crude oil in artificial sea water medium, which 91.80% of crude oil was degraded by this bacterium. Even the degradation rate was very good, however, the metabolites might still dangerous for environmental, thus the optimization is still needed such as modification of mixed cultures. *B. subtilis* may be mixed with *R. pickettii* [17]. Besides, *B. subtilis* can be mixed with some brown-rot fungi (BRF), such as *Gloeophyllum trabeum*, *Daedalea dickinsii*, and *Fomitopsis pinicola* as well as white-rot fungi (WRF) such as *Pleurotus ostreatus*, *Pleurotus eryngii*, and *Ganoderma lingzhi*. Mixed culture *B. subtilis* and BRF had been reported to enhance DDT degradation as well as dyes decolorization [29–32].

Conclusions

The bacterium *B. subtilis* is capable of degrading petroleum in the high-salinity water. The optimum recovery value of crude oil degradation by the bacterium *B. subtilis* was obtained by octadecadienoic acid compound gave a percentage recovery of 8.20% (91.80% degradation) after 7 days of incubation. This result indicated that the *B. subtilis* culture has the ability to degrade crude oil spill in simulated sea water.

Conflict of Interest

The authors declare that there is no conflict of interest.

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