EFFECT OF BIOSURFACTANT PRODUCED BY *BACILLUS* IN OILY WASTEWATER DEGRADATION

By
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ABSTRACT

Liquid waste from oil industry activities has potential cause environmental pollution. These liquid wastes, containing hydrocarbon and heavy metals, are mostly toxic. Therefore, biotechnology by means of biological treatment can be applied for decomposing the toxic liquid wastes. The biosurfactant production from some microorganisms can support hydrocarbon degradation. The objective of this study is to examine the crude biosurfactant that is extracted from the selected Bacillus which was precipitated by using methanol on acid moiety. The crude biosurfactant extract were tested to support hydrocarbon degradation. Three species of Bacillus used in this experiment were compared based on their respective biosurfactant production. The results showed that the percentage of hydrocarbon degradation on liquid waste from refinery by the three Bacillus species were 90.23% (Bacillus subtilis), 88.72% (Bacillus licheniformis), and 73.43% (Bacillus laterosporus). The concentration of remaining oil after 28 days was 20.44 mg/L, 23.38 mg/L, and 54.87 mg/L, respectively. The decrease of COD were 84.90%, 84.04%, and 80.68%, respectively, and the COD value after 28 days treatment were 165 mg/L, 174 mg/L, and 211 mg/L.

Key words: biodegradation, biosurfactant, wastewater, Bacillus

I. INTRODUCTION

Wastewaters produced from oil industries have considerable content of hydro-carbons and other compounds that contain sulphur, nitrogen, oxygen, and mineral, including heavy metals. Most of these compounds are toxic in character, therefore, these compounds have potential to cause negative impact to the environment and should therefore be treated before being discharged to the environment.

There are basically three methods of wastewater treatment based on composition and characteristics of wastewater: physical, chemical, and biological methods. Usually physical treatment is only used at the beginning of the process, it is usually followed by chemical treatment which gives better result, however it is very expensive due to its requiring special chemical reagent. In addition, chemical treatment process causes other pollutions. The oil content in wastewater dominant would generally cover the wastes surface and obstruct sunshine and oxygen penetration into the water. The best method for oily wastewater degradation is by biological treatment, because this process is more economic, efficient, and environmental friendly (Eckenfelder 1989).

Surfactant is usually used to decrease oil content. The phenomenon of oil degradation via surfactant addition is based on its capability to decrease surface tension. The surfactants commonly used are synthetic surfactants produced by organic chemical reactions (Zajic et al. 1977). Biosurfactant is a surfactant compound produced by microorganisms. It is environmental friendly, easy to degrade in a biological manner. *Bacillus* is one of the types of bacteria that can produce biosurfactant and also can degrade hydrocarbon. The objective of this research is to study the effect of using bacteria from genus of *Bacillus* produced biosurfactant to support the biodegradation of oily wastewater process.

II. MICROORGANISMS

The microorganisms such as bacteria, fungi, yeast, and actinomycetes can live in hydrocarbon. Usually bacteria is the best microbes used to degrade hydrocarbons. Bossert and Bartha (1984) have discovered 22 genus of bacteria that can live in crude oil sites. The majority of bacteria in crude oil are *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Coryneform*, *Flavobacterium*, *Nocardia*, and *Pseudomonas.*

Indigenous microorganisms in oily waste are capable to degrade hydrocarbons due to their secreted enzyme that acts as a biocatalyst for biodegradation process (Bartha and Atlas 1987). If there are no indigenous microorganisms existing or present only in limited amount in crude oil sites, the addition of exogenous microorganisms into oily matter can help to degrade the hydrocarbon more efficiently (Rocha et al 2001).

III. BIOSURFACTANT

Biosurfactants are biological amphiphatic compounds consisting of hydrophilic and hydrophobic domains. Microorganisms have been reported to produce several classes of biosurfactants such as glycolipids, lipopeptides, phospholipids, neutral lipids or fatty acids and polymeric biosurfactants (Cooper and Kosaric, 1981). These compounds are produced during the growth of microorganisms on water-soluble and water immiscible substrates. The surfactants accumulate in the interface between different polarity phases such as water and oil, air and water, or fluid and solid substances. This process, based on the capability of surfactants to decrease the surface tension through arrangement of the molecules until interaction is formed between hydrogen bond and hydrophobic-hydrophilic structure. Margaritis et al. (1979) found that addition of surfactant concentration will eventually reach CMC (critical micell concentration). In this condition, the characteristics of fluid such as its surface tension, osmosis tension, viscosity, density, and electrical conductivity will change unexpectedly. In micelle, surfactant molecules have tendency to form a spherical agregate with hydrophobic group gathered in center and the hydrophilic group at the outside.

IV. OILY WASTEWATER AND BIODEGRADATION

Crude oil refineries produce oil fuels. In the process, they produce also wastewater that contains hydrocarbons. These hydrocarbons will cover the surface of waters and interfere biota life. Almost all of the hydrocarbons will be degraded by photooxidation process and also by microorganisms. Hydrocarbons in wastewater potentially cause damage, because:

- Oil layer in the surface will cut off oxygen diffusion from air to water, thus minimizing soluble oxygen in water.
- Oil layer also cut off the entrance of sunshine into water and interferes in plant photosynthesis.
- It also causes danger to water birds, because the oil will stick and cover the feathers.

Oily wastewaters need special treatment before being discharged to the environment. The hydrocarbon biodegradation rate is influenced by physical and chemical characteristics of the oil and also by in-situ microorganisms. This capability of hydrocarbon degradation and converting it into non-toxic substances depends on the enzyme that is produced by the microorganisms.

To preserve the growth and hydrocarbon degradation capability by microorganisms, we can control some factors such as nutrient which has essential elements (nitrogen and phosphor) also oxygen and temperature (-2^{o}C to 70^{o}C). Paraffin is easier to degrade than naphthenic and aromatic compounds.

V. METHODOLOGY

A. *Preparation*

The bacteria (*Bacillus*) that was used in these experiment is drawn from a collection of LEMIGAS

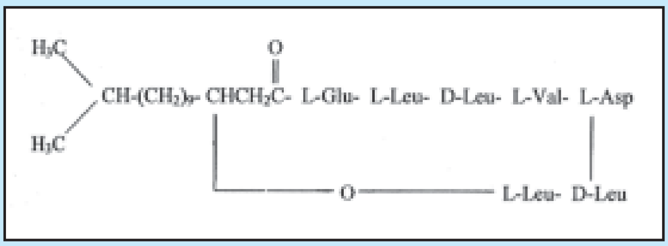

Figure 1
Structure of surfactin that produced from *B. subtilis*

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Laboratory of Microbiology, especially from BLCC (Biotechnology Lemigas Culture Collection). LEMIGAS became member of FORKOMIKRO (2007), which stands for Communication Forum for Indonesian Culture Collection Curators was established in 20th March 1996. The selected species are of *Bacillus* namely *B. subtilis*, *B. licheniformis*, and *B. laterosporus*. All of those can degrade hydrocarbons, but *B. laterosporus* does not produced biosurfactant. Those biosurfactant producing bacteria were refreshed and cultivated during seven days in mineral salt. The substrate was added with yeast extract, glucose, and crude oil. Every day (24 hours), the surface tension and the optical density (OD) were examined by using Processor Tensiometer K12 Kruss and spectrophotometer, respectively. The OD analysis was used to observe the growth of those bacteria, because OD value shows bacteria population. The oily wastewater samples were collected from Indonesian Refinery.

B. *Biosurfactant Extraction*

The process of biosurfactant extraction as follows, one volume of aquadest was added into the cultivation/inoculum, and then centrifuged at 10.000 rpm speed for 20 minutes to separate the biomass. Supernatant liquid is controlled at acid condition (pH=3) by addition of HCl and then precipitated by using three volume of methanol, let one night at -15^{o}C temperature. Crystal of biosurfactant, formed by centrifugation in 10.000 rpm, was washed with methanol, and dried at ambient temperature. Crude extract of biosurfactant was obtained and examined by using gas chromatography (HPLC Waters). Biosurfactant production observation were examined every day until seven day treatments. The surfactin from Sigma® was used as a standard that produced from *B. subtilis*. The structure of surfactin is shown in Figure 1.

C. *Oily Wastewater Biodegradation*

The experiment was conducted in batch condition during 28 days with observations after (1, 2, 3, 6, 10, 15, 21, and 28 days). Oily wastewater was enriched with urea and KH_2PO_4 as nitrogen and phosphor source (N:P=5:1) for the growth of the bacteria. As a control, oily wastewater without bacteria is added was used. The parameters analyzed include: total petroleum hydrocarbon (Concawe 1/72 and FTIR), COD (chromate oxidation), microbial population (TPC method), and acidity (pH meter). The hydrocarbon concentration analysis was examined after 0, 6, and 28 day treatment, using Gas Chromatography, of Hewlett Packard 5890 series II, with FID as the detector.

VI. RESULT AND DISCUSSION

A. *Optical Density and Surface Tension*

Based on the OD analysis of *B. subtilis* and *B. licheniformis* (Figure 2), the exponential phase of *B. licheniformis* had been reached in the first day while *B. subtilis* on the second day. The stationary phase for both species was reached on the fourth day. The growth of *B. subtilis* is better than *B. licheniformis*

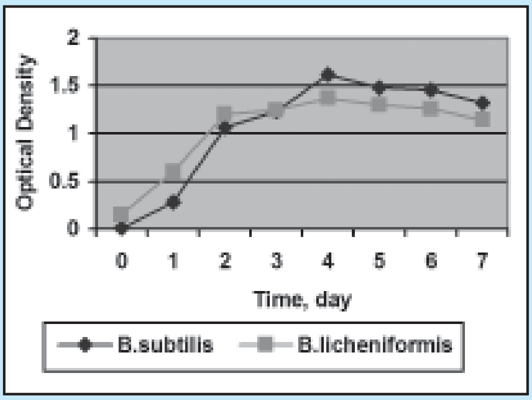

Figure 2
Optical density of *B. subtilis* and *B. licheniformis* in 7 days cultivation

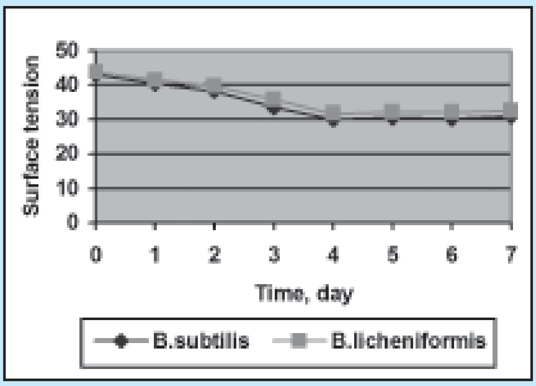

Figure 3
Surface tension of *B. subtilis* and *B. licheniformis* in 7 days cultivation

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and the highest OD was reached at 1.6 for *B. subtilis* and 1.4 for *B. licheniformis*.

The growth of the two bacteria, *B. subtilis* and *B. licheniformis*, are related to surface tension (see Figure 3). Surface tension decreases at the beginning of the first cultivation until the fourth day, it means that biosurfactant had been produced. The linear curve in surface tension showed that biosurfactant stops being produced after four days, due to the stationary phase from bacterial growth (Figure 2). The lowest surface tension for *B. subtilis* is 30.01 mN/m and *B. licheniformis* is 31.69 mN/m.

B. *Biosurfactant Production*

Biosurfactant production by *B. subtilis* and *B. licheniformis* is shown in Figure 4. The production increases from the beginning cultivation until six days for *B. subtilis* with the maximum biosurfactant production of 1.4700 g/L and *B. licheniformis* still increases on the seventh days (1.3980 g/L). Biosurfactant production is related with the growth of both bacteria.

The analysis of biosurfactant concentration by using surfactin as a standard and HPLC Waters as the instrument. The chromatograms of biosurfactant produced by *B. subtilis* and *B. licheniformis* are identical with surfactin from Sigma®. The surfactin concentration in biosurfactant that was produced by both bacteria is shown in Table 1.

C. *Oily Wastewater Biodegradation*

Table 2 shows that hydrocarbon degradation (TPH) during 28 day treatment by *B. subtilis* gives the highest result (90.23%), followed by *B. licheniformis* (88.72%) and *B. laterosporus* (73.43%). The biosurfactant that is produced by *B. subtilis* and *B. licheniformis* supported hydrocarbon degradation. All bacteria could degrade hydrocarbons by more than 50% in 10 days. TPH remains at 28 day by *B. subtilis* (20.44 mg/L) and *B. licheniformis* (23.38 mg/L) were accomplished the Standard Quality of Wastewater (max 25 mg/L), but not with *B. laterosporus* (54.87 mg/L).

The decrease of COD concentration during 28 day treatment has the same pattern with TPH concentration (Figure 5). COD concentration from *B. subtilis* and *B. licheniformis* were accomplished with Standard Quality of Wastewater (max 200 mg/L), but not with *B. laterosporus* (211 mg/L).

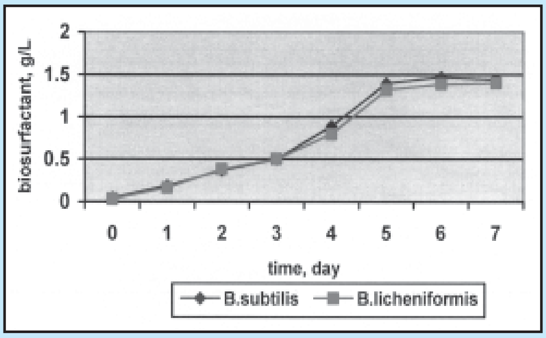

Figure 4
Biosurfactant production by *B. subtilis* and *B. licheniformis* in 7 days cultivation

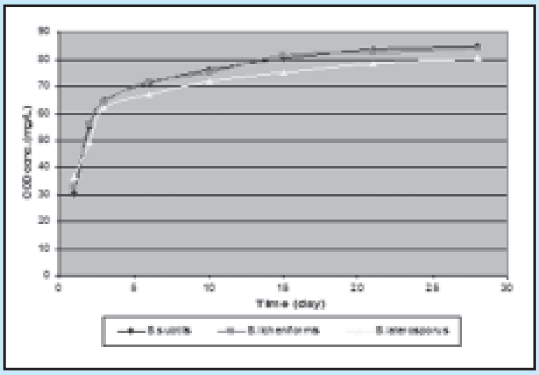

Figure 5
Analysis of COD in oily wastewater by *B. subtilis*, *B. licheniformis*, and *B. laterosporus*

Table 1
Surfactin concentration in biosurfactant produced by *B. subtilis* and *B. licheniformis*

No.	Bacteria	Conc. of biosurfactant (surfactin, ppm)
1.	*B. subtilis*	24.99
2.	*B. licheniformis*	23.65

During 28 day treatment of oily wastewater degradation, pH decreased until 15 days, but the value was still in the pH range for bacterial growth (Figure 6). The pH value was accomplished with Standard Quality of Wastewater.

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Table 2
Analysis of TPH in oily wastewater by *B. laterosporus*, *B. subtilis*, and *B. licheniformis*

Day	*B. laterosporus*		*B. subtilis*		*B. licheniformis*		Control	
	TPH, mg/L	TPH (%)	TPH, mg/L	TPH (%)	TPH, mg/L	TPH (%)	TPH, mg/L	TPH (%)
0	209.07	-	209.16	-	206.51	-	208.13	-
1	165.71	20.69	159.53	23.97	147.31	27.84	206.13	0.61
2	131.96	36.95	115.13	43.78	118.93	41.68	205.00	0.97
3	117.13	43.74	99.82	52.02	105.13	49.06	205.33	1.38
6	88.27	57.84	71.78	65.75	80.78	60.83	204.47	2.45
10	72.98	64.60	45.84	77.55	56.91	72.41	200.60	3.43
15	63.00	69.70	28.02	86.51	34.89	83.05	196.60	4.99
21	58.27	72.00	23.33	88.88	25.40	87.80	191.67	6.11
28	54.87	73.43	20.44	90.23	23.38	88.72	191.53	8.42

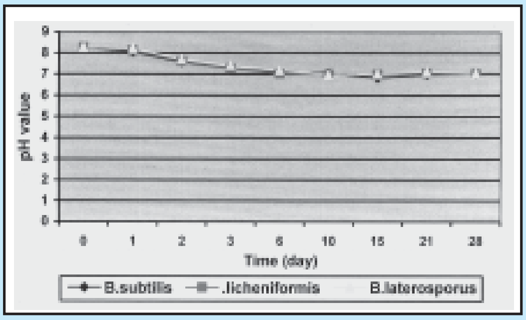

Figure 6
Analysis of pH in oily wastewater by *B. subtilis*, *B. licheniformis*, and *B. laterosporus*

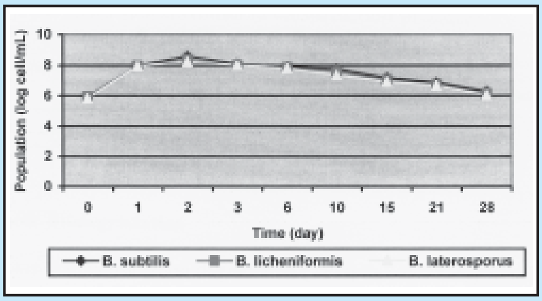

Figure 7
Analysis of population in oily wastewater by *B. subtilis*, *B. licheniformis*, and *B. laterosporus*

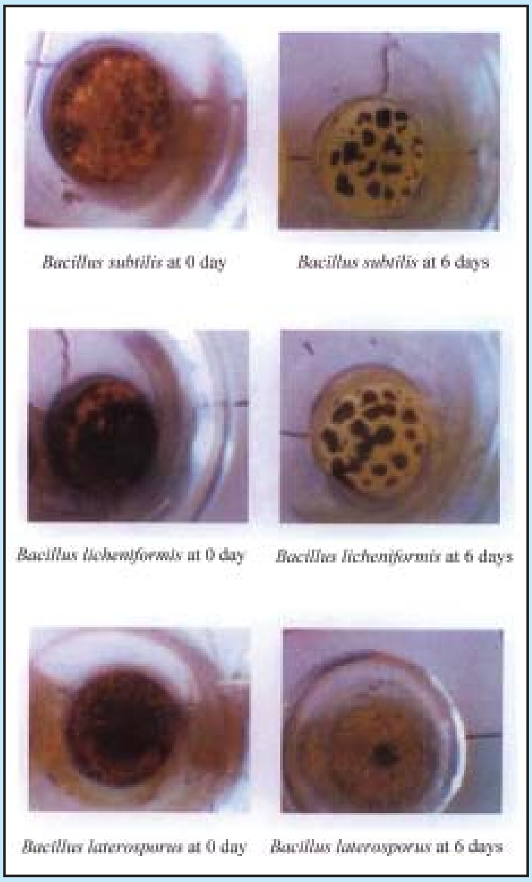

Figure 8
Oily wastewater biodegradation by *Bacillus* at 0 and 6 days

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Table 3
Hydrocarbon concentration using GC after 0, 6, and 28 day's treatment

Atom C Numb	*B. laterosporus*			*B. subtilis*			*B. licheniformis*		
	0 day (mg/L)	6 days (mg/L)	28 days (mg/L)	0 day (mg/L)	6 days (mg/L)	28 days (mg/L)	0 day (mg/L)	6 days (mg/L)	28 days (mg/L)
6	1.28	-	-	1.28	-	-	1.28	-	-
7	13.36	0.07	-	13.36	-	-	13.36	-	-
8	13.94	2.07	-	13.94	-	-	13.94	-	-
9	12.86	2.75	-	12.86	0.08	-	12.86	0.32	-
10	12.90	4.40	-	12.90	0.95	-	12.90	2.34	-
11	11.79	4.56	-	11.79	2.22	-	11.79	3.97	-
12	12.31	5.11	-	12.31	4.67	-	12.31	4.70	-
13	13.19	6.29	1.15	13.19	5.27	-	13.19	6.26	-
14	19.90	8.09	2.33	19.90	7.05	-	19.90	8.93	0.74
15	16.04	8.28	5.91	16.04	7.90	0.58	16.04	8.90	1.23
16	16.22	7.70	6.26	16.22	8.48	0.89	16.22	9.21	1.46
17	16.83	7.62	6.57	16.83	7.86	1.28	16.83	8.13	1.84
18	13.13	7.08	5.41	13.13	7.54	1.75	13.13	7.20	2.59
19	10.50	6.36	3.96	10.50	5.17	2.15	10.50	5.78	2.70
20	9.81	5.75	3.64	9.81	3.72	2.35	9.81	4.05	2.46
21	7.32	3.48	3.17	7.32	2.98	2.41	7.32	3.15	1.71
22	2.11	2.53	3.02	2.11	1.98	1.95	2.11	1.79	1.54
23	1.61	1.78	2.73	1.61	1.71	1.75	1.61	1.71	1.73
24	1.34	1.37	2.35	1.34	1.42	1.43	1.34	1.42	1.45
25	0.96	1.11	2.24	0.96	1.03	1.19	0.96	1.01	1.21
26	0.79	0.83	2.02	0.79	0.80	0.87	0.79	0.85	0.90
27	0.48	0.56	1.77	0.48	0.50	0.77	0.48	0.54	0.76
28	0.29	0.35	1.47	0.29	0.33	0.69	0.29	0.36	0.67
29	0.10	0.13	0.84	0.10	0.12	0.38	0.10	0.13	0.39
Total	209.07	88.27	54.87	209.07	71.78	20.44	209.07	80.78	23.38

The growth of the three bacteria showed that exponential phase reached on second day, especially for *Bacillus subtilis*, followed by *Bacillus licheniformis*, and then *Bacillus laterosporus* (Figure 7).

D. *Hydrocarbon Composition*

Based on the GC analysis, the hydrocarbons consist of C6 (hexane) up to C29 (nonakosane). At six day treatment (incubation) it was found that C6 up to C8 were completely degraded by *B. subtilis* and *B. licheniformis*, however for *B. laterosporus* C7 and C8 have not degraded yet. *B. subtilis* degrades hydrocarbon more easily compared to the other bacteria. After 28 days, *B. subtilis* could completely degrade hydrocarbons up to C14 and *B. licheniformis* up to C13. However *B. laterosporus* that cannot give biosurfactant production, only completely degraded hydrocarbons up to C12 (see Table 3 and Figure 8). In Table 3, hydrocarbon concentration remaining after 28 days are as follows: by *B. laterosporus* 54.87 mg/L (C13-C29), by *B. subtilis* 20.44 mg/L (C15-C29), and by *B. licheniformis* 23.38 mg/L (C14-C29). In general, hydrocarbons of higher

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than C15 cannot be completely degraded by the three bacteria mentioned. Degradation of hydrocarbons that contain more than C15 carbon atoms requires continued incubation or treatment. It related with Yani et al. (2003), hydrocarbons in wastewater that contain C4 up to C40 carbon atoms, cannot be totally degraded by microorganisms.

VII. CONCLUSION

The conclusions of this study are:

1. The hydrocarbon degradation by *B. subtilis* is better than *B. licheniformis.*
2. *B. subtilis* and *B. licheniformis* which produced biosurfactant are more effective to support the process of biodegradation.
3. Biosurfactant production by *B. subtilis* and *B. licheniformis* are identical with surfactin from Sigma®.
4. All bacteria of *Bacillus* genus could degrade hydrocarbons by more than 50% in 10 days.
5. At 28 day treatment, three bacteria of *Bacillus* genus could completely degrade hydrocarbons up to C12.

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