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Original Article

The potency of *Micrococcus* sp. L II 61 bacteria as oil sludge cleaning agent

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Abstract

This research aimed to reveal the ability of *Micrococcus* sp. L II 61 bacteria that was isolated from Pegirian Surabaya as oil cleaning agent. This is an experimental research to detect the presence of biosurfactant and lipase enzyme in culture supernatant of *Micrococcus* sp. L II 61 with aliphatic hydrocarbon (cooking oil) as a substrate growth. Biosurfactant production was evaluated by measuring the surface tension of supernatant using tensiometer du Nouy and measuring the emulsification activity value using diesel oil as hydrocarbon test. Lipase enzyme was detected by measuring lipolitic activity value of crude enzyme (culture supernatant) by using p-nitrofenil palmitic (p-npp) as a substrate test. Data were analyzed descriptively. The results showed that *Micrococcus* sp. L II 61 produced biosurfactant with surface tension decreasing of culture supernatant up to 30.27 ± 1.17 mN/m compared than aquadest and value of hydrocarbon emulsification activity (AE 1 hour) up to 20.24 ± 0.68 %. Culture of *Micrococcus* sp. L II 61 after 16 hours incubation have a lipolytic activity 33.53 ± 0.14 U/mL at pH 7 and 37 ºC. Supernatant of *Micrococcus* sp. L II 61 100% (v/v) give the highest percentage of oil sludge solubility, i.e. 86.38 ± 2.39%. *Micrococcus* sp. L II 61 is a highly potential to be developed as oil sludge cleaning agent.

Keywords: *Micrococcus sp. L II 61, biosurfactant, lipase enzyme, oil sludge, cleaning agent*

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Introduction

Oil sludge is a precipitated oil in the form of mud or black paste mixed with soil, gravel, water and other materials. Oil sludge is still a major problem faced by the oil industry. Accumulation of oil sludge in tanks of oil will cause a decrease in oil storage capacity and can speed up the process of thinning the oil tank. Efforts to clean up or removal of oil sludge which not lifted by conventional pump are the focus of research being developed (Banat and Rancich, 2009).

Accumulation of oil sludge in the ground can be also lower the quality of the environment, especially on the soil fertility, ground water quality and environmental health (Ni'matuzahroh et al., 2009). During this time, oil sludge cleanup efforts that considered effective are the use of surfactant. However, synthetic surfactants can cause problems for the environment because of its resistant, difficult to degrade biologically, and toxic when it accumulated in a natural ecosystem (Fiechter, 1992).

Biotechnological applications by using microbial products to replace chemical surfactants which have the same function as surfactants are starting to be developed. Bacterial products potentially used in dissolving oil waste consist of biosurfactant and lipase enzyme. Lipase will hydrolyze the oil which is not soluble in water into a water soluble product, whereas biosurfactant decreases the surface tension and interfacial tension in the space between the water and oil (Ni'matuzahroh et al., 2012).

Exploration of enzyme producing bacteria from slaughterhouse waste by Fatimah et al., (2011) has obtained *Micrococcus* sp. L II 61 isolate. *Micrococcus* sp. L II 61 produced protease and lipase enzymes and had a high lipolytic index. Thus, it is interesting to test the potency of *Micrococcus* sp. L II 61 in dissolving oil sludge.

This study focused on the measurement of lipolytic activity of lipase enzyme and biosurfactant production on culture supernatant of *Micrococcus* sp. L II 61, and the potency of culture supernatant of *Micrococcus* sp. L II 61 in dissolving oil sludge. This research may be useful in the oil sludge cleanup efforts that environmentally friendly.

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Method

Materials

Materials used in this research include *Micrococcus* sp. L II 61 isolates, oil sludge, Bushnell-Haas mineral salts medium, p-NPP, Na_2CO_3, Nutrient Broth (NB), cooking oil, diesel oil, and distilled water.

Culture of *Micrococcus* sp. L II 61

Micrococcus sp. L II 61 was grown in 50 mL of Bushnell-Haas mineral media plus 2 % (v/v) oil in 250 mL bottle culture. Media was sterilized and inoculated with 5 % (v/v) culture of *Micrococcus* sp. L II 61 grown on Nutrient Broth, with a value of optical density up to 0.5 at λ= 650 nm. Bacterial cultures were incubated in a shaker at 120 rpm, room temperature for 50 hours. The culture was centrifuged at a speed of 9,000 rpm, in 4 °C for 15 min. Supernatant of *Micrococcus* sp. L II 61 was used to test the lipase enzyme activity and the presence of bio-surfactant.

Enzyme Activity Assay

Lipase activity of *Micrococcus* sp. L II 61 supernatant was measured every 4 hours until 48 hours of culture incubation time. It was measured quantitatively using the spectrophotometric method with p-nitrophenyl palmitate

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(p-NPP) as a substrate. Eppendorf was filled with 700 mL solution of 0.503 mM p-NPP in phosphate buffer pH 7 and plus 300 mL of supernatant. The mixture was then incubated in a waterbath at 37 ºC for 30 minutes. Eppendorf was removed from the waterbath and then added 100 mL of 0.2 M Na_2CO_3 solution. The p-nitrophenol formed is marked in yellow, and then was measured with a UV-Vis spectrophotometer at λ = 410 nm. The absorbance value obtained will then be used to set the value of lipase activity using a standard curve that was created earlier (Pereira et al., 1997). Measurement of lipase activity was repeated 3 times. The best incubation time which gave the highest value of lipolytic activity was used as a reference in crude lipase production. Value of lipase activity is expressed in U/mL.

Biosurfactant Detection Test

Biosurfactant production by *Micrococcus* sp. L II 61 on a culture supernatant was detected by measuring the surface tension value (mN/m) using a Du Nouy tensiometer and emulsification activity (%) test on hydrocarbons (diesel oil) after 1 and 24 hours of incubation time. The procedure for measuring biosurfactant production referred to Pruthi and Cameotra (1997). Growth media without bacteria was used as control.

Preparation of Synthetic Surfactant Tween-20 at a Concentration Equal to The CMC

Synthetic surfactant Tween-20 weighed as much as 0.11 g/L to achieve the same concentration of CMC (Critical Micelle Concentration) was dissolved in phosphate buffer (pH=7). This solution was characterized by measuring the surface tension values (mN/m) and emulsification activity (%) on hydrocarbon (diesel oil). Tween-20 (=CMC) is used as a control in testing the solubility of oil sludge using *Micrococcus* sp. L II 61 supernatant.

The Solubility Test of Oil Sludge

Oil sludge solubility test was performed in the laboratory scale with 5 treatments and 1 control. The total volume was 8 ml. Variations of each treatment is described as follows.

- Control (T), which is 0.20 mL of oil sludge + 7.80 mL synthetic surfactant Tween-20 at a concentration equal to the CMC.
- Treatment 1 (A), which is 0.20 mL of oil sludge + 7.80 mL of distilled water.
- Treatment 2 (Ea), which is 0.20 mL of oil sludge + 1 mL supernatant of *Micrococcus* sp. L II 61 + 6.80 mL of distilled water (12.5 % v/v supernatant/ total volume)
- Treatment 3 (Eb), which is 0.20 mL of oil sludge + 2 mL supernatant of *Micrococcus* sp. L II 61 + 5.80 mL of distilled water (25.0 % v/v supernatant/ total volume)
- Treatment 4 (Ec), which is 0.20 mL of oil sludge + 3 mL supernatant of *Micrococcus* sp. L II 61 + 4.80 mL of distilled water (37.5 % v/v supernatant/ total volume)
- Treatment 5 (E8), which is 0.20 mL of oil sludge + 7.8 mL supernatant of *Micrococcus* sp. L II 61 (100 % v/v supernatant/ total volume)

Each treatment was performed with 3 replications. Mixture of each treatment was vortexed at room temperature (27-30ºC) for 15 minutes. Oil sludge dissolved in the liquid phase was then centrifuged at a speed of 9,000 rpm for 15 min at 4°C.

Measurement of the Oil Solubility in The Water Phase

A total of 1 mL sample of the liquid phase from the centrifugation was poured in the filter paper coated aluminum foil, and then dried at a temperature of 51-55 ° C for six hours. Solubility of oil sludge was measured by the quantity of oil trapped in the filter paper. The dried filter paper was weighed and its weight was recorded, and expressed as Wt. All procedures were performed free of fat. Each treatment was performed in the oil sludge solubility test, always accompanied by a blank. Blank was a supernatant solution without the addition of oil sludge. Blank weight of each treatment was recorded as Wb.

Percentage of Oil Sludge Solubility

The percentage value of oil sludge solubility of each treatment can be calculated using the following formula referred to Eaton et al., (2005):

$$\% \text{ solubility of oil sludge} = (Wot - Wb) / Wop \times 100\%$$

Wot = weight of dissolved oil sludge, Wb = weight of blank treatment (g), Wop = weight of oil sludge tested (g)

While, the weight of the dissolved oil sludge is calculated using the following formula referred to Michel (2011):

$$Wot = (Wt - Wo) \text{ X Total Volume}$$

Wot = weight of dissolved oil sludge (g/total volume), Wt = weight of filter paper + dissolved oil sludge (g/mL), Wo = weight of filter paper (g)

Results

Lipase activity of *Micrococcus* sp. L II 61 Supernatant

Micrococcus sp. L II 61 produces the lipase enzyme in its growth. Lipase activity in the culture supernatant increased up to 8 hours of incubation and fluctuated until the end of the incubation (48 hours). A highest activity was obtained at 16 hours of incubation time, (31.16 U/mL). The value of lipase activity of *Micrococcus* sp. L II 61 supernatant of every 4 up to 48 hours of incubation time is presented in Figure 1.

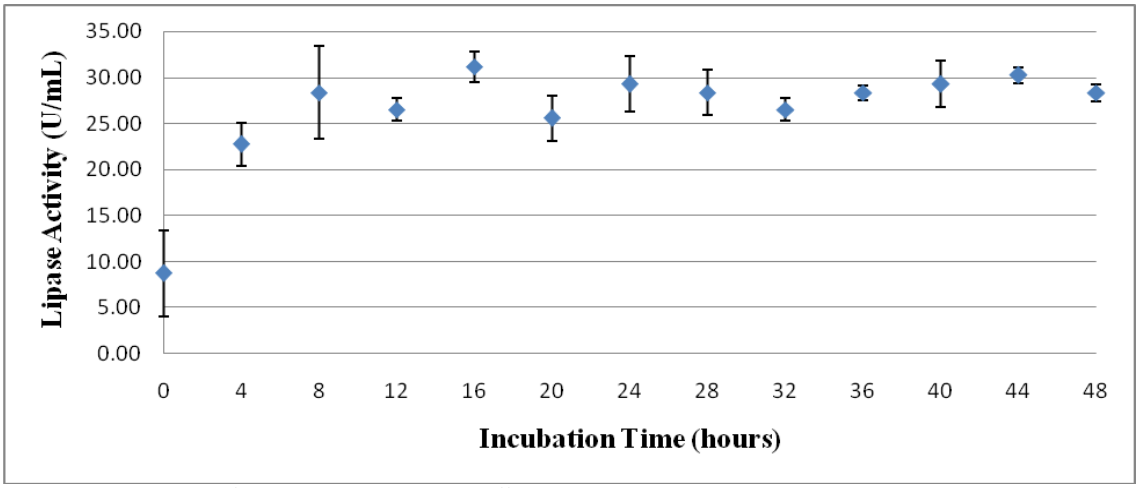

Figure 1. Lipase activity of *Micrococcus* sp. L II 61 on different incubation time

Table 1. Emulsification activity and surface tension value of *Micrococcus* sp. LII 61 supernatant at 16 hours of incubation time

Types tested	Emulsification activity (AE) on diesel oil (%)		Surface tension (mN/m)
	1 hour	24 hours	
Supernatant of *Micrococcus* sp. L II 61	20.24 ± 0.68	17.31 ± 1.19	41.73 ± 1.17
Growth media	0.00 ± 0.00	0.00 ± 0.00	63.20 ± 2.61
Tween – 20	35.5 ± 1.03	32.25 ± 2.19	56.76 ± 0.28

Emulsification Activity and Surface Tension Value of *Micrococcus* sp. L II 61 Supernatant and Tween - 20

Micrococcus sp. L II 61 also produces biosurfactant on hydrocarbon substrates. Biosurfactant production by *Micrococcus* sp. L II 61 proved from hydrocarbon emulsification activity and decreasing value of the surface tension of culture supernatant, is presented in Table 1. The value of emulsification activity of *Micrococcus* sp. L II 61 supernatant on diesel oil after 1 hour and 24 hours were 20.24 ± 0.68 % and 17.31 % ± 1.19 % respectively. These values are lower than the value of emulsification activity of synthetic surfactant Tween-20 at a concentration equal to the CMC after 1 hour and 24 hours on diesel oil, where the values are 35.5 ± 1.03 % and 32.25 ± 2.19 %.

The surface tension of *Micrococcus* sp. L II 61 supernatant was 41.73 ± 1.17 mN/m. There are decreases of 21.47 ± 1.02 and 30.27 ± 1.17mN/m when it is compared to growth media and distilled water. Decreasing of surface tension values more than 10 mN/m indicates that bacteria could potentially produce biosurfactant (Francy et al., 1991). The surface tension of *Micrococcus* sp. L II 61 supernatant was lower than Tween-20 at a concentration equal to the CMC (56.76 ± 0.28 mN/m), with a decrease in surface tension against distilled water at 15.24 ± 0.28 mN/m and phosphate buffer pH 7 at 14.18 ± 0.28 mN/m.

Solubility of Oil Sludge at Various Treatments

Oil sludge solubility using various concentration of *Micrococcus* sp. L II 61 supernatant is presented in Figure 2. Supernatant of *Micrococcus* sp. L II 61 can dissolve the oil sludge. The results of statistical tests using one-Way ANOVA (α 0.05) shows that variation of supernatant have a significant influence on the solubility of oil sludge (%). Duncan test results showed that there is a significant difference between treatment groups on solubility of oil sludge (%).

Treatment (E8) with the addition of 100 % (v/v) *Micrococcus* sp. L II 61 supernatant containing crude lipase and biosurfactant had the highest percentage on the oil sludge solubility up to 86.38 ± 2.39 %. Contrary, treatment A (distilled water) cannot dissolve oil sludge. Meanwhile, treatment which only added to 12.5% (v/v) supernatant (Ea) had a percentage of oil sludge solubility higher than in the distilled water, which is 27.28 ± 1.30 %.

Control treatment (T) using Tween-20 at a concentration equal to the CMC had a percentage of oil sludge solubility up to 32.19 ± 1.46%. The results of oil sludge solubility by Tween-20 did not differ significantly with 25% (v/v) *Micrococcus* sp. L II 61 supernatant. This indicates that the *Micrococcus* sp. L II 61 supernatant potentially replace the use of synthetic surfactants as oil sludge cleaning agent.

Discussions

Oil sludge contains both aliphatic and aromatic hydrocarbons. Bacteria grown on oil substrates was known to be able to produce enzyme and biosurfactant which have an important role in the degradation of hydrocarbons (Leahy and Colwell, 1990; Ni'matuzahroh et al., 2012a). *Micrococcus* sp. L II 61 is one of the hydrocarbonoclastic bacterial isolates that potentially produces lipase enzymes and biosurfactant. In certain circumstances, lipases are able to catalyze the hydrolases of ester bonds of inter phase between insoluble substrate phase and the water phase (Ghosh et al., 1996). Biosurfactant as amphiphatic compounds are capable to emulsify and solubilize the hydrocarbon in the water phase.

Supernatant of *Micrococcus* sp. L II 61 contains both crude lipase and biosurfactant. Lipase enzyme

catalyzes ester bond hydrolysis of aliphatic hydrocarbon, a water-insoluble compound, into smaller particles and increase the solubility of oil sludge. Meanwhile, biosurfactant serves to maintain the oil particles in the water phase. Combination of lipase and biosurfactant in the solubilization of hydrocarbons and insoluble compounds are widely used for industrial applications such as oil and detergent industries (Jurado et al., 2007). Interaction between biosurfactant and lipase and protease enzymes affected the solubility of oil sludge (Ni'matuzahroh et al., 2012a). The ability of *Micrococcus* sp. L II 61 bacteria to produce enzymes and biosurfactant will increase the prospects of application in the field.

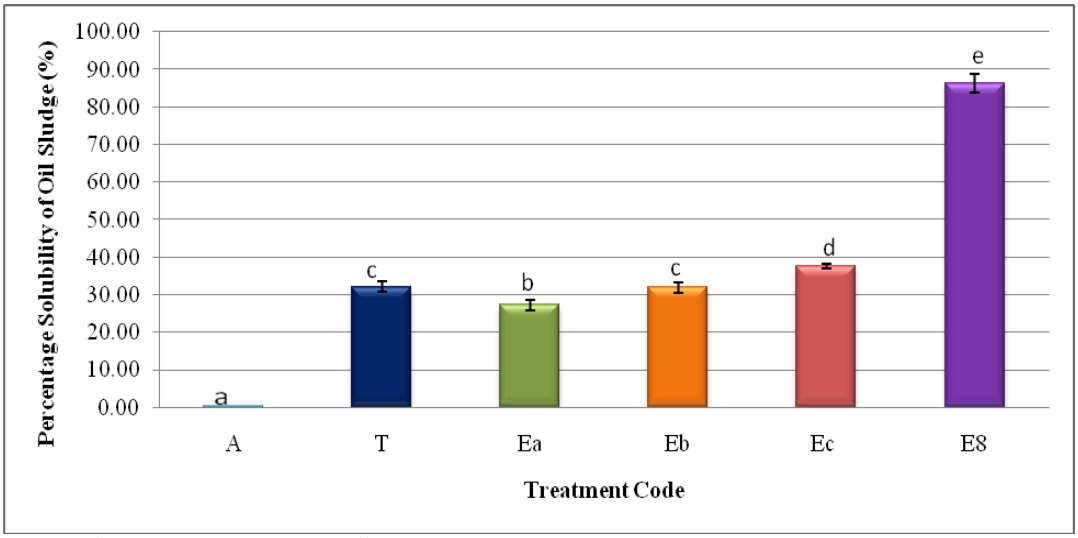

Figure 2. Percentage of oil sludge solubility on the different treatment. Notes: A = Aquadest, T = Tween-20, Ea = Supernatant 12,5 % (v/v), Eb = Supernatant 25 % (v/v), Ec = Supernatant 37,5 % (v/v), E8 = Supernatant 100 % (v/v). The average percentage of oil sludge solubility followed by a different letter notation indicates a significant difference (α 0.05). Notation letter by Duncan test results.

Genus *Micrococcus* was known to have the ability to utilize a variety of unusual substrates such as pyridine, herbicides, chlorinated biphenyls and oils. *Micrococcus* has the ability to detoxify or biodegradation of many other hydrocarbon pollutants in the environment. Some species of *Micrococcus* also began to be applied in the oil biodegradation research, such as *Micrococcus luteus* (Hamza et al., 2009) and *Micrococcus* variance (Khan and Sigh, 2011). *Micrococcus* sp. is also capable of producing neutral phospholipids and fatty acids. Supernatant of *Micrococcus* sp. has a crude lipase with the active center of serine-imidazole, therefore, similar to the esterolytic enzyme (Lawrence et al., 1967).

This study successfully demonstrated that the product of *Micrococcus* sp. L II 61 can be used as an alternative as a oil sludge solving agent, replacing the use of synthetic surfactants. Supernatant of *Micrococcus* sp. L II 61 had solubilization activity of oil sludge better than the *Actinobacillus* sp. bacteria at only 85 % (Ni'matuzahroh et al., 2012b). *Micrococcus* sp. L II 61 has the potential to be used as an oil sludge cleaning agent because of its ability to dissolve the oil sludge. *Micrococcus* sp. L II 61 also potentially be used as hydrocarbon degrading bacteria because of its function to produce the lipase enzyme and biosurfactant at once. Optimization of lipase production of *Micrococcus* sp. L II 61 is also the focus of research to be developed. Potency of *Micrococcus* sp. L II 61 on biodegradation of hydrocarbons and oil pollution bioremediation are still under further investigation.

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