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5th International Conference and Workshop on Basic and Applied Sciences (ICOWOBAS 2015)

Surabaya, Indonesia
16–17 October 2015

Editors
Moh. Yasin and Professor Dr. Sulaiman W. Harun

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Preface: 5th International Conference and Workshop on Basic and Applied Sciences.

Foreword from Chairman of 5th ICOWOBAS 2015

Ladies and Gentleman,

The 5th International Conference and Workshops on the Basic and Applied Sciences (ICOWOBAS) is organized of existing collaborations between Airlangga University, Universiti Teknologi Malaysia and Salahaddin-Hawler University (Erbil, Iraqi Kurdistan) in order to promote the development of sciences and their prospect of application in industry and medical devices. The program of this activity are the scientific program involves the presentation of the paper and poster in the area of chemistry, biology, physics, mathematics and their applications. It also conducts the workshop program was presented the current issues in optical instrumentation. Thus invited many participants as academic researchers, scientists, industrial professionals, government officers, students and other participants. The meeting intends to bring together researcher, scientists and scholars to exchange and share their experiences, new ideas, research novelties in related fields and discuss the practical challenges and the solutions adopted.

The AIP proceedings hold the full papers presented at the 5th ICOWOBAS. The conference took place in Surabaya (Indonesia) at the Garuda Mukti Room, Kampus C Universitas Airlangga, October 16th - 17th, 2015, and the workshop was conducted at the Faculty of Science and Technology, Airlangga University, October 15th, 2015.

The conference included: Prof. Dr. Retna Apsari (Universitas Airlangga, Indonesia) as invited speaker. In total, we received 152 abstracts for oral and 29 posters, and 46 full paper selected in AIP proceeding. As the acceptance rates illustrates the competition is stiff, and the accepted submission reflected high rates of reviewer enthusiasm. By design, these papers have been through peer-review process and they are almost accepted.

ICOWOBAS is a lot of work. We could not have done it without help from many people. We would especially like to thank: Scientific board of ICOWOBAS, for inviting us to chair the meeting; The Rector of Airlangga University, for supporting us to conduct the conference; our colleagues in the Faculty of Science and Technology, Airlangga University, for their support in the conference; the local committee, for organizing and handling the conference; the many reviewers, for providing professional reviews; our sponsor: Vitalong C and DGHE through Airlangga University funding.

Surabaya, February 2016

Dr. Moh. Yasin

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Oil Removal from Petroleum Sludge using Bacterial Culture with Molasses Substrate at Temperature Variation

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Abstract. *The study aims to reveal the potency of biosurfactant-producing bacterial culture with molasses as substrate growth in releasing oil from the petroleum sludge at temperature variations. Bacteria used consisted of (Acinetobacter* sp. *P2(1), Pseudomonas putida T1(8), Bacillus subtilis 3KP and Micrococcus* sp. *L II 61). The treatments were tested at 40°C, 50°C and 60 °C for 7 days of incubation. Synthetic surfactant (Tween 20) was used as a positive control and molasses as a negative control. Release of petroleum hydrocarbons from oil sludge was expressed in percentage of oil removal from oil sludge (%). Data were analyzed statistically using the Analysis of Variance (α = 0.05) and continued with Games-Howell test. The kinds of bacterial cultures, incubation temperature and combination of both affected the percentage of oil removal. The abilities of Bacillus subtilis 3KP and Micrococcus* sp. *LII 61cultures in oil removal from oil sludge at the temperature exposure of 60°C were higher than Tween 20. Both of bacterial cultures grown on molasses can be proposed as a replacement for synthetic surfactant to clean up the accumulation of oil sludge in a bottom of oil refinery tank.*

Keywords: *Bacterial, Biosurfactant, Molasses, Oil sludge, Oil removal, Temperature*

1 Introduction

Activity in the petroleum industry which conducted continuously will generate waste in the form of oil sludge. Oil sludge contains some complex hydrocarbon compounds that are not easily degraded. Accumulation of oil sludge in oil distribution pipelines will result sedimentation of oil tank bottom and clogging the tank. This oil sludge will affect the operational capacity of the oil storage tanks and accelerate the corrosion process [1,2]. Oil sludge was categorized into hazardous waste and toxic compounds. Accumulation of oil sludge in the environment can also contaminate soil and water environment [3].

Efficient and effective method for treating of oil sludge is needed in efforts to overcome the oil sludge problem in environment [4,5]. Generally, there are three ways conducted to overcome oil precipitate of oil sludge, such as : mechanical, chemical and biological ways. Mechanically, the oil sludge is taken by physically using manpower, and this is very harmful to human health [1]. Chemically, oil sludge was cleaned using cleaning compounds, such as organic solvents and surfactant compounds [6,7]. During this time, the use of synthetic surfactants still have a negative impact on the environment because it is resistant, difficult broken down biologically, and is very toxic when accumulated in a natural ecosystem [8]. Biologically, it was conducted by increasing the solubilisation of hydrocarbon in oily sludge using the activity of living organisms through the degradation activity of microorganism [9]. This method is more advantageous because it can be produced quickly and are not harmful to the environment.

The use of microorganism in oil cleaning treatment has being developed in the world [1]. This microbes known as hydrocarbonoclastic microorganism can produce the biosurfactant to help the hydrocarbon solubility of oil sludge. Biosurfactants can lower surface tension, increases the solubility of hydrophobic compounds and provide ease of microbes to degrade hydrocarbons [10]. The effectiveness of oil cleaning treatment depend on potency of microorganism, concentration of microorganism, microbial growth factor (such as nutrient, oxygen,

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temperature, pH, salinity, pressure), type and concentration of active compound, and incubation time [5,11,12,13].

The use of molasses as a nutrient for hydrocarbonoclastic bacteria to produce biosurfactant starts much attention. While, efforts in enhancing of oil sludge solubility by using biosurfactant-producing bacterial culture on molasses with incubation temperature variation is still rarely done. Application of bacterial culture using molasses substrate on oil cleaning treatment will be more efficient and prospective to treat oil sludge.

This article reveal the effectiveness of culture of hydrocarbonoclastic bacteria isolated from oil contaminated soil in Indonesia with molasses substrate in releasing oil hydrocarbons from oil sludge on variation of temperature.

2 Experimental methods

2.1 Preparation of bacterial stock

The bacteria used include: (*Acinetobacte*r sp. P2 (1), *Pseudomonas putida* T1 (8), *Bacillus subtilis* and *Micrococcus* sp 3KP. LII 61). These bacteria are a collection of microbiology laboratories, Faculty of Science and Technology, Universitas Airlangga. The bacteria are stored in Nutrient Agar medium. Petroleum sludge was obtained from oil refinery industry in Kalimantan, Indonesia.

2.2 Preparation of bacterial growth media

The bacteria were grown on synthetic mineral medium (AMS) with the addition of molasses. Mineral medium composition per g/liter in accordance with [5] composition, comprising: (NaCl, $NaNO_3$, $CaCl_2$, $MgSO_4.7H_2O$, KCl, $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $MnSO_4.H_2O$, $NaMoO_4.2H_2O$, H_3BO_3, $CoCl_2.6H_2O$, K_2HPO_4, H_2PO_4, $(NH_4)_2SO_4$, $BaSO_4$, NaOH, HCl). The molasses was used as a carbon source. Molasses was obtained from sugar factory in East Java-Indonesia.

2.3 Bacterial growth on molasses

Each bacterium was grown on Nutrient Broth medium and incubated for 24 hours. Subsequent, bacterial culture turbidity value is set to reach a value of OD = 0.5 at λ = 650 nm. 500 mL size bottle culture was filled with 172.8 mL mixture of Synthetic Mineral Water (SMW) and 2% (v/v) molasses substrate pH 7. Mixed media was sterilized by autoclave. Then, to the medium, it was added 9.6 mL of stock KH_2PO_4 and K_2HPO_4 and 9.6 mL of stock $FeSO_4.7H_2O$ and 4% (v/v) bacterial inoculums (*Acinetobacte*r sp. P2 (1), *Pseudomonas putida* T1 (8), *Bacillus subtilis* or *Micrococcus* sp 3KP. LII 61) as much as (8 mL) which has a value of OD = 0.5 at λ = 650 nm. So, the total volume of the mixture of the test is 200 mL. Bacterial cultures were incubated in a shaker at 120 rpm for 4 days [5].

2.4 Oil removal test using bacterial culture with variation of temperature

The release of oil from oil sludge was carried out using agitation in the variation of incubation temperature for 7 days. Oil removal test was conducted by first preparing a volume of 250 ml bottle cultures as much as 54 bottles that had previously been sterilized by autoclave for 20 minutes at a temperature of 121°C pressure of 1 atm. Oil sludge was weighted as much as 1 gram, and added in the culture bottles. Next, prepared the culture products of each bacteria that were incubated for 4 days. Each of 30 ml bacterial culture is inserted into the sterile bottle which already contains oil sludge. Treatments were incubated at 40°C, 50°C and 60°C at 120 rpm for 7 days in an incubator shaker. Tween 20 was used as the positive control and negative controls in the form of molasses. Tests were carried out on the same control with the treatment.

2.5 Enumeration of bacterial cell

Bacterial growth was evaluated by total plate count (TPC) method . Increasing the number of bacterial cells per ml per incubation time for each treatment is expressed in units (CFU / ml). 1 mL sample from a dilution series was included in Petri dishes, added ± 15 mL NA, homogenize, and incubated for 24 hours. The number of bacteria grown in a Petri dish media NA was multiplied by 1/dilution factor.

2.6 Determination of percentage value and effectiveness value of oil removal

The percentage of oil release from oil sludge was detected using gravimetric methods. The liquid phase of the culture treatment was separated from the solid phase in different bottles containing oil sludge. Washing hydrocarbons attached to the bottle was enhanced by rinsing using N-hexane. 15 ml of hydrocarbon liquid phase containing oil was taken and extracted using n-hexane at a ratio of 1: 2 ie culture: 30 ml n-hexane. Extraction is done gradually by added 10-10-10 ml n-hexane (shaken for ± 15 minutes) Next, the water phase and an organic phase were formed, separated and placed on each different bottle. Then, the organic phase was evaporated for ± 20 minutes, with a boiling point of n-hexane at 60-70°C to obtain % of dissolved oil content.

The percentage of oil removal was calculated using the following formula:

% Oil removal = (weight of oil soluble) / (initial oil weight) x 100%

The effectiveness of oil removal was calculated using the following formula:

$$\text{Efectivenesss} = \frac{\text{Test sample} - \text{Control}\ (-)}{\text{Control}\ (+) - \text{Control}\ (-)}\ \text{x}\ 100\%$$

2.7 Data analysis

All experiments were conducted in triplicates. Results were evaluated statistically using ANOVA and continued by Games-Howell test.

3 Results and Discussion

3.1 Bacterial growth of different kinds of treatments

Molasses can be used as a medium for growth and biosurfactant production media for the four tested bacteria ((*Acinetobacte*r sp. P2 (1), *Pseudomonas putida* T1 (8), *Bacillus subtilis* and *Micrococcus* sp 3KP. LII 61). Growth of the four bacteria on molasses media for 4 days of incubation was evaluated by the increase the number of bacterial cells (CFU / ml) in each culture. Each of these bacteria give a different response, indicated by the number of bacterial cells are varied, ranging from $1{,}4\text{x}10^{8}$, $2{,}49\text{x}10^{15}$, $2{,}92\text{x}10^{16}$, $2{,}04\text{x}10^{2}0$ CFU / ml respectively for (*Acinetobacte*r sp. P2 (1), *Pseudomonas putida* T1 (8), *Bacillus subtilis* and *Micrococcus* sp 3KP. LII 61) bacteria.

The presence of bacteria in the oil sludge was evidenced by the presence of bacteria on positive control culture (Tween 20) and a negative control (molasses) added to the oil sludge. Increasing the number of bacteria also occur in four bacterial cultures tested. Treatment of temperature variation (40°C, 50°C, 60°C) for 7 days of incubation has provided growth response that is different for each treatment (Figure 1). *Acinetobacte*r sp. P2 (1) bacteria undergo growth inhibition with increasing temperature. Meanwhile, the three bacteria *Pseudomonas putida* T1 (8), *Bacillus subtilis* 3KP and *Micrococcus* sp. LII 61) can survive up to 50°C and inhibited at 60°C.

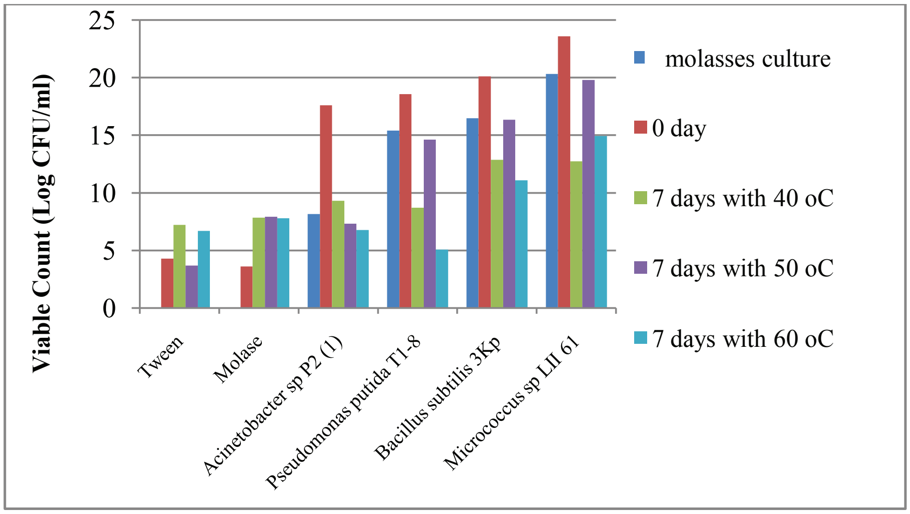

Figure 1. The number of bacteria in each culture containing molasses and oil sludge. Tween20 (positive control) and molasses (negative control)

The number of bacteria on the 7 day of incubation tends to decline compared to the beginning of incubation (0 days). Bacterial growth is assumed to have passed the log phase before the end of the incubation period of 7 days. The growth curves of bacteria are not reported in this article. The growth rate decreased when the supply of substrate and oxygen decreases, changes in pH, and the accumulation of metabolic substances that inhibit the growth. Survival ability of a microbe in a growth medium is affected by the ability to compete for the nutrients and type of interactions with other microbes [14]. Synergistic between indigenous bacteria in oil sludge with exogenous bacteria in molasses culture largely determines the ability of a bacterial culture to remove oil from oil sludge [5].

3.2 The influence of the type of bacterial culture on the release of oil from petroleum sludge

Type of bacterial cultures on molasses provides different capabilities to remove oil from oil sludge (Figure 2). Culture of *Acinetobacte*r sp. P2 (1), *Pseudomonas putida* T1 (8), and *Bacillus subtilis* 3KP have the ability to remove oil up to 17.02%; 14.87% and 14.54% respectively. Bacterial culture of *Micrococcus* sp LII 61 has a higher percentage value of oil removal than the three other bacterial culture that is equal to 21.64%. However, it is still lower than Tween 20 in the amount of 28.21%. Tween 20 is able to provide higher percentage value of oil removal than any bacterial culture. Tween 20 is a synthetic surfactant that proven to increase the oil solubility of the oil sludge deposits.

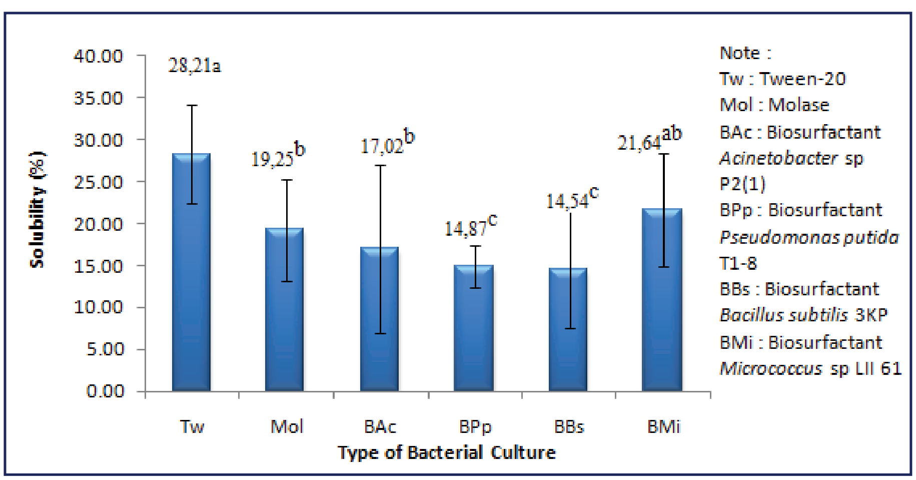

Figure 2. Percentage of oil removal on different types of bacterial culture.

Micrococcus sp. LII 61 has the potential to increase the solubility of the oil because these bacteria can produce biosurfactant. In addition, it is also known as enzyme-producing bacteria. Interaction between biosurfactant and hydrocarbon-degrading enzymes will increase the ability of the release of oil from oil sludge [12]. Capability of *Micrococcus* sp. LII 61 culture of dissolving the petroleum hydrocarbon because of the synergy of its biosurfactant performance and enzymes product. *Micrococcus* is also known can utilize a variety of substrates and has the ability to detoxify or degrade of many other hydrocarbon pollutants in the environment [12]. Thus, it can accelerate oil degradation process of petroleum hydrocarbons. According to [15], a bacterium which has the adaptability to petroleum hydrocarbons will show a higher rate of biodegradation.

3.3 Influence of temperature on oil removal

Temperature influenced percentage of oil release from oil sludge (Figure 3). Statistical test result of temperature variation on the percentage of oil removal using Two-Way ANOVA test Univariate showed a less significantly than the value (α) 0.05 is 0.000. Thus, Ho is rejected, which means that the incubation temperature affect the oil release percentage. Games-Howell test results showed that the temperature of 50°C did not differ significantly with temperature of 60°C, but differ significantly with temperature of 40°C.

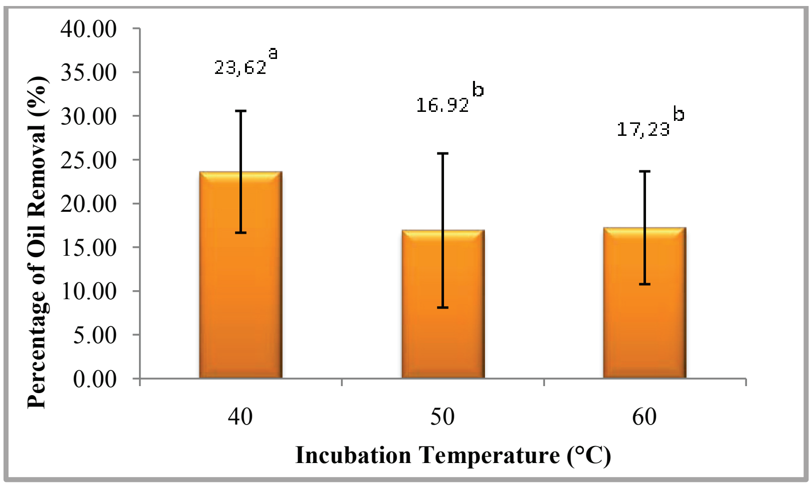

Figure 3 Percentage of oil removal in variation of temperature

The percentage of oil release at 40°C with an incubation time of 7 days up to 23.62%. Meanwhile, at a temperature of 50°C and 60°C with an incubation time of 7 days, the average percentage of oil release is equal to 16.92% and 17.23%. Differences in oil release results of oil sludge can be caused by several possibilities, first, that the temperature is the optimum temperature for growth of tested bacteria. Second, diversity in temperature can alter certain metabolic processes of bacterial cell. Third, the change of functional groups surfactant due to changes in temperature can improve the performance of surfactant [16]. Temperature stability is also one biosurfactant properties that can affect the effectiveness and commercialization biosurfactant, in addition to the stability of the pH and salinity [17].

3.4 The percentage of oil removal by a combination of the type of bacterial culture and incubation temperature

The combination of types of bacterial culture (*Acinetobacter* sp. P2 (1), *Pseudomonas putida* T1-8, *Bacillus subtilis* 3KP, and *Micrococcus* sp. L II 61) with a variation of incubation temperature (40°C, 50°C and 60°C) produced the different percentage of oil removal (Figure 4). Molasses is used as a negative control and a synthetic surfactant (Tween 20) was used as a positive control.

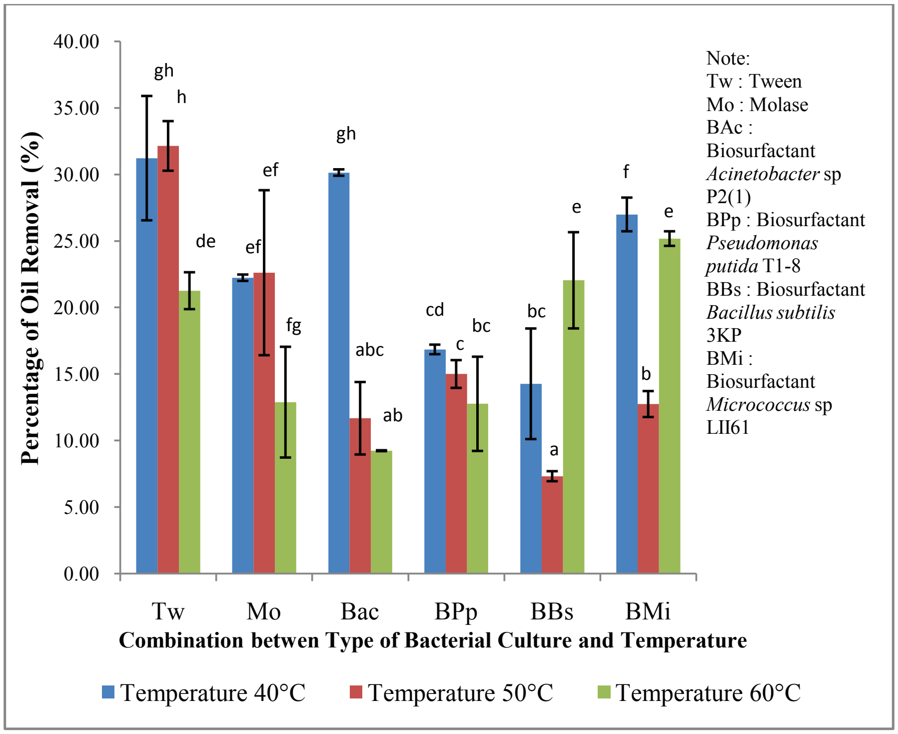

Figure 4. Percentage of Oil Removal on the Combination of Treatments

The types of combined treatment effected on the percentage of oil removal. The effectiveness of different combinations of bacterial culture can be shown from the percentage value of oil removal comparing with the synthetic surfactant (Tween-20). Results of statistical analysis with Two Way ANOVA Univariate tests showed a significance value of less than (α) 0.05 is 0,000. So, that Ho were rejected which showed no effect of the combination of types of bacterial culture and incubation temperature on the oil removal (%). Games-Howell test showed no significant difference between treatments. The combination of types of bacterial culture *Acinetobacter* sp. P2 (1) at a temperature of 40°C has a solubility values were not significantly different with Tween-20 at 40°C. Similarly, the type of bacterial culture *Micrococcus* sp. LII 61 40°C showed no significant difference with the Tween-20 at a temperature of 40°C. Thus, the type of *Micrococcus* sp. LII 61 bacterial culture was prospectively in replacing Tween 20.

Bacterial culture *Micrococcus* sp. LII 61 at 60°C with 7 days of incubation have high solubility percentage that is equal to 25.18%. This correlates with the number of bacterial cells from the growth curve *Micrococcus* sp LII 61 which is higher than the three types of others bacterial culture. In contrast to research conducted by [5] that the culture of *Micrococcus* sp. LII 61 at 60°C with 7 days of incubation capable of releasing oil 36,2%.

Acinetobacter sp. P2 (1) bacterial culture in this study showed a lower of bacterial cells, but the culture of *Acinetobacter* sp. P2 (1) effective in releasing oil at a temperature of 40°C is equal to 30.14%. This result was higher than [5], which only reached 10.56% at 50°C with an incubation time of 7 days. Differences in solubility results mentioned above because of the tested bacterial culture consist of whole cell and its biosurfactant which was produced along 4 days incubation. The incubation time of 4 days is the optimum time to produce biosurfactant [13]. This treatment used agitation 120 rpm with an incubation time of 7 days. Research [5] used molasses and bacterial inoculums added simultaneously at the treatment for 7 days with agitation of 120 rpm.

The solubility of hydrocarbon from oil sludge can also be caused by the concentration of surfactant produced by a type of bacteria. Based on previous research, by [13], mentioned that the biosurfactant product of *Micrococcus* sp. LII 61 using molasses media on the 4th day of incubation have ≥CMC (*Critical Micelle Concentration*) value. Thus, the bacterial culture *Micrococcus* sp. LII 61 can affect the results of the solubility of the petroleum hydrocarbon in oil sludge. This, supported with [16] statement that the role of solubilization occurs when biosurfactant above the CMC value. At this concentration of biosurfactant, molecules associate to form micelles, which dramatically increase the solubility of the oil. In addition, the synergy of cooperation between the biosurfactant and the lipase enzyme produced by bacteria serves to catalyze the hydrolysis of the ester bond in the lipid substrate which is not water soluble [18]. Thus, the oil will break down into smaller particles that will be taken and retained by biosurfactant in the liquid phase. Agitation (shaking) is also capable of affecting the results of the percentage of oil sludge solubility, because agitation could increase bioavailability hydrocarbon oils by microbes and microbial products.

The ability of oil removal by bacterial culture *Micrococcus* sp LII 61 at 60°C higher than the Tween-20, although it did not differ significantly, ie by 25.18%. Not only the culture of *Micrococcus* sp. LII 61, *Bacillus subtilis* culture 3KP at temperature of 60°C also has a percentage value higher than the Tween-20 at temperature of 60°C is equal to 22.25%. Thus, *Bacillus subtilis* culture 3KP also prospectively to replace Tween 20, although not significant to the culture of the *Micrococcus* sp LII 61 at 60°C and *Acinetobacter* sp. P2 (1) at temperature of 40°C.

Culture of *Acinetobacter* sp. P2 (1) shows a higher solubility value at a temperature of 40°C. However, at 60°C, solubility activity of the bacterial culture decreased by 19.80%. This shows that the culture of *Acinetobacter* sp. P2 (1) is only effective at temperatures less than 40°C. Petroleum tank washings generally require high temperatures to process oil sludge solubility. Bacterial culture *Micrococcus* sp. LII 61 with a temperature of 60°C is effective at dissolving oil. This values are not significant to the Tween-20 at temperature of 60°C.

Effectiveness value of the combination of types of bacterial culture and incubation temperature showed different results. Of the four combinations of types of bacterial culture and incubation temperature, bacterial culture of *Bacillus subtilis* 3 KP with a temperature of 60°C gives the value of the effectiveness of 109.3% while, kind of a bacterial culture *Micrococcus* sp. LII 61 gives the value of the effectiveness of 146.83%. The effectiveness value of these bacterial cultures is more than 100%. Thus, both types of bacterial culture is likely to replace the Tween-20. However, based on the value of effectiveness of both types of bacterial culture with a temperature of 60°C, the type of culture the bacteria *Micrococcus* sp. LII 61 shows the effectiveness of a higher value. Thus, the type of bacterial culture *Micrococcus* sp. LII 61 with molasses substrate in an incubation temperature of 60°C more prospective to replace the Tween-20 and has the opportunity to assist the washing process the oil in the oil tank bottom.

4 Conclusion

Bacterial culture containing molasses can be used to remove oil from the oil sludge. The types of bacterial culture affect the ability of oil removal. Temperature affects the percentage of oil removal. Bacterial culture can be used as an oil cleaning agent up to 60°C even though there is a reduction in activity. Cultures of *Bacillus subtilis* 3KP and *Micrococcus* sp. LII 61 are prospective for use as oil cleaning agent of oil sludge

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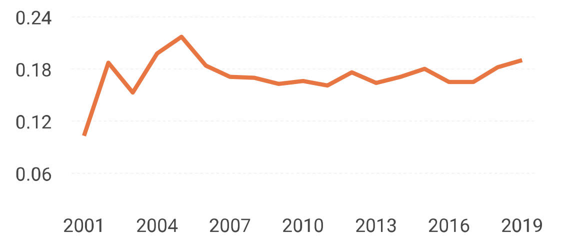

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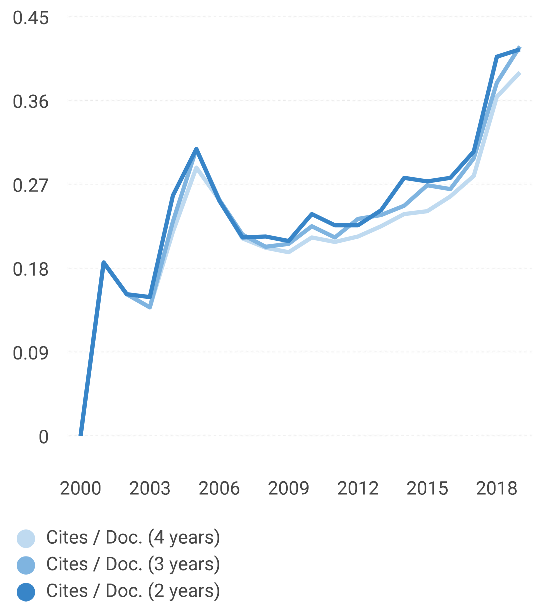

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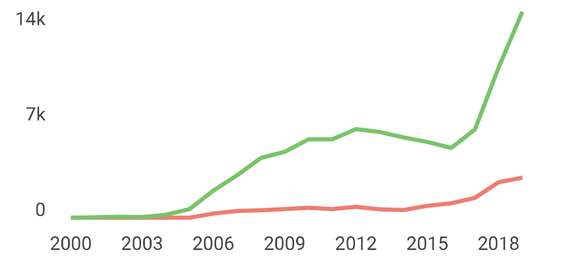

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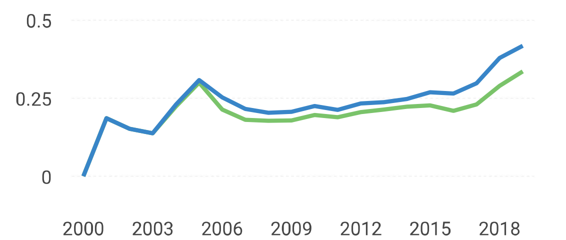

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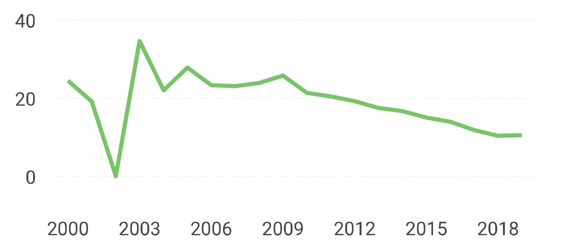

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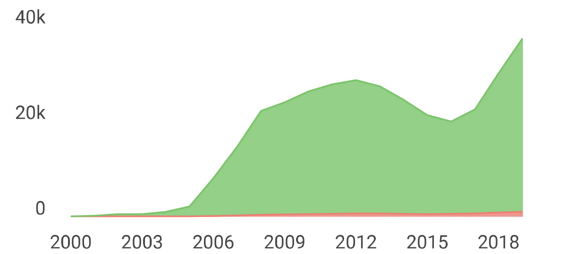

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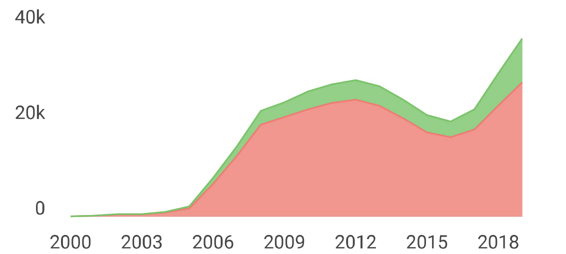

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