J. Appl. Environ. Biol. Sci., 2(12)645-651, 2012

ISSN 2090-4215
Journal of Applied Environmental and Biological Sciences
www.textroad.com

The Solubility of Oil Sludge by *Bacillus subtilis 3KP* Biosurfactant and Crude Enzyme

Ni'matuzahroh[1,*], Hidayatul Muna, Fatimah[1], Sri Sumarsih[2]

(1) Departement of Biology, Faculty of Science and Technology, Airlangga University
(2) Department of Chemistry, Faculty of Science and Technology, Airlangga University
Kampus C Unair , Jln. Mulyorejo, Surabaya - Indonesia

ABSTRACT

The objective of this research was to find out the effect of biosurfactant, crude enzymes (lipase and protease), and the combination of both toward the solubility of oil sludge. Biosurfactant was produced by *Bacillus subtilis* 3KP. Crude enzymes were produced by bacteria isolated from organic wastes area. The solubility of oil sludge was measured using a filtration method. Data obtained were analysed statistically using One-way ANOVA and Duncan test (p=0.05). The analysis result showed that biosurfactant and or crude enzyme gives an influence toward oil sludge solubility. Percentage of oil sludge solubility by biosurfactant was about 41%. A combination of biosurfactant and crude lipase gave lower sludge solubility percentage, it was about 38%. And a combination of biosurfactant and crude protease gave higher oil sludge solubility percentage, it was about 64%. The highest oil sludge solubility was resulted by a combination of crude lipase and protease as much as 89%, but that result was not significantly different with oil sludge solubility resulted by lipase enzyme alone (85%).The percentage of oil sludge solubility was higher than Tween-20 as synthetic surfactant (51%). The result of this research showed that *Bacillus subtilis* 3KP biosurfactant, crude enzyme, and the combination of both could be suggested to be of a great alternative as a natural cleaning agent of oil sludge in oil storage tank, which it has a friendly environmental characteristic.

Keywords: solubility of oil sludge, biosurfactant of *Bacillus subtilis* 3KP, crude lipase, crude protease

INTRODUCTION

One of the major problems facing the oil industry is oil sludge produced during the processing of crude oil. Accumulation of oil sludge on the bottom of oil storage tanks can cause a diminution operational capacity and accelerate the corrosion process on a tank. This is of course will inflict harm to petroleum industry. So, it requires a cleaning effort or depuration of oil sludge from petroleum storage [1].

Surfactant is an amphiphatic agent that can be used to lift oil sludge from bottom of oil storage tanks. Synthetic surfactant is commonly used for cleaning purposes. However, discharging some synthetic surfactant can cause problems for the environment because of its resistant characteristic to be degraded biologically. It is also highly toxic when it accumulates in an ecosystem [4].

Surfactant produced by microorganism, commonly called as biosurfactant, is proposed to replace the use of synthetic surfactant in oil sludge cleaning. Biosurfactant is friendly for environment and it can be produced using renewable substrate with a cheaper cost. *Bacillus subtilis* 3KP is a bacterial collection of microbiology laboratory, Faculty of Science and Technology, Airlangga University, Indonesia. This bacteria is very potential to produce biosurfactant on a molasses substrate. The biosurfactant is classified as bioemulsifier compound [11]. In the previous study, it has also been obtained potential bacteria producing enzyme from organic waste. These bacteria and its products were started investigated for various uses, among others is for reprocessing oil waste.

Recently, some researchers developed the use of biosurfactant and enzyme to increase effectiveness of oil sludge solubility. Mixing supernatant of *Bacillus subtilis* IAM 1213 culture which producing biosurfactant with *Aeromonas sp. NKB26c* as lipase and protease enzyme producer also can be used as oil biocleaner [17]. This product has a higher efficiency in oil cleaning of ceramic tile of factory buildings compared to a single use of *Bacillus subtilis* IAM 1213 supernatant or *Aeromonas sp. NKB26c*.

This research aims to know the potency of *Bacillus subtilis* 3KP biosurfactant, lipase and protease enzymes that were produced by Indonesian local isolate in oil sludge solubility. This research is expected to be useful in handling oil sludge in petroleum industry and also can be used for pre treatment in oil waste processing in environment.

***Corresponding Author:** Ni'matuzahroh, Department of Biology, Faculty of Science and Technology, Airlangga University, Surabaya Indonesia. Email: nimatuzahroh@yahoo.com

MATERIALS AND METHODS

Biosurfactant production

Bacillus subtilis 3KP strain was isolated from "Kali Donan" river, Cilacap, Central Java [10]. The bacteria was cultivated on Nutrient Broth (NB) with NaCl addition and incubated for 24 hours at 30 ^{o}C. Afterwards, the culture suspension was used as inoculants in microbial cultivation. Culture of bacteria were prepared in OD=0.9 at $\lambda_{610nm.}$

About 2% (v/v) of *Bacillus subtilis* 3KP culture suspension was then inoculated to mineral salts medium developed by [13]. The flasks contain molasses 3% v/v as carbon source. The cultures were placed on incubator shaker at 120 rpm, 30 ^{o}C for 4 days. The biosurfactant medium was separated from bacterial cell by centrifugation at 9000 rpm, 4^{o}C during 15 minutes.

Biosurfactant extraction

Bacillus subtilis 3KP biosurfactant was extracted from supernatant by 60% saturation $(NH_4)_2SO_4$ precipitation. The remained product was lyophilized using freeze dryer to obtain crude biosurfactant, and then weighed. The product will be used in oil sludge solubility test [11].

Preparation of biosurfactant solution

Biosurfactant was determined at CMC (Critical Micelle Concentration) value. This concentration was about 16 g/L [11]. Biosurfactant solution was made by dissolving crude biosurfactant into buffer phospat, pH 7. Biosurfactant solution was then characterized by measuring surface tension value (mN/m) and emulsification activity towards kerosene as hydrocarbon tested. These characterizations must be done to measure the strength of biosurfactant.

Preparation of Tween-20 solution

Synthetic surfactant solution of Tween-20 used in oil sludge solubility test was determined at CMC value. CMC concentration of Tween-20 was about 0.11 g/L. Biosurfactant solution was made by dissolving Tween-20 into buffer phospat, pH 7. Tween-20 solution was then characterized by measuring surface tension value (mN/m) and emulsification activity (%). These characterizations must be done to measure the strength of Tween-20 synthetic surfactant.

Production of crude protease

Crude protease was produced by *Bacillus* sp. RPH 2.3 isolated from organic waste of abattoir in "Pasar Pacar Keling" Surabaya, East Java. The bacteria was cultivated on Nutrient Broth (NB) and incubated for 24 hours at 30 ^{o}C. Culture suspension was prepared in OD=0.5 at λ_{650nm} and used as inoculants. About 1% (v/v) culture suspension was inoculated into *Bushnell-Haas mineral salts medium* which added with 2% (w/v) of skim milk. *Bushnell-Haas mineral salts* contained per liter distilled water, KH_2PO_4 (1 g), K_2HPO_4 (1 g), NH_4NO_3 (1 g), $MgSO_4.7H_2O$ (0,2 g), $FeCl_3$ (0,05 g), $CaCl_2.2H_2O$ (0,02 g). The cultures were placed on incubator shaker at 120 rpm for 24 hours at 30 ^{o}C. Crude protease was obtained by centrifugation at 9000 rpm, 4^{o}C during 15 minutes. Crude protease was then measured its proteolytic activity, surface tension, and emulsification activity.

Proteolytic activity assay

Proteolytic activity was assayed semi-quantitatively by measuring the diameter of inhibition zone (mm) formed in skim milk Bushnell-Haas agar plate after 24 hours of incubation time. Bushnell-Haas agar plate contains 2% (w/v) of skim milk, Bushnell-Haas mineral salts medium, and agar powder. Crude protease was dropped on sterile paper disk, and then laid in Petri dish containing skim milk Bushnell-Haas agar plate and was incubated at 30 ^{o}C. Hallo zone formed around the paper disk after 24 hours was measured using calliper. The proteolytic activity was determined toward casein as substrate tested.

Production of crude lipase

Crude lipase was produced by *Actinobacillus sp.* P3-7 strain isolated from traditional crude oil exploration, Wonocolo, Bojonegoro, East Java. The bacterial was cultivated on Nutrient Broth (NB) and incubated for 24 hours at 30 ^{o}C . Afterwards, culture suspension was prepared in OD=0.5 at λ_{650nm} and used as inoculants. About 2% (v/v) culture suspension was inoculated into NB medium containing 2% of palm oil. The cultures were incubated at 120 rpm, 30 ^{o}C for 24 hours. The crude enzyme was obtained by centrifugation at 9000 rpm, 4^{o}C during 15 minutes. The crude enzyme was then measured its lipolytic activity, surface tension, and emulsification activity.

Lipolytic activity assay

The lipolytic activity of *Actinobacillus* sp. P3-7 crude enzyme was assayed semi-quantitatively by measuring diameter of inhibition zone (mm) formed after 24 hours on Rhodamine-B agar plate [6]. Lipase of *Actinobacillus* sp. P3-7 was dropped on sterile paper disc, then laid in Rhodamine-B agar plate medium and was incubated at 30 ^{o}C. Diameter of hallo zone formed around paper disk after 24 hours was measured using calliper.

The lipolytic activity was assayed toward *p*-nitrofenil palmitat (*p*-NPP) as a substrate by means of modificated method of [12]. Enzyme activity was determined by using spectrophotometer UV-Vis at $\lambda_{410\ nm}$. One unit (U) of lipolytic activity represents the amount of lipase enzyme hydrolyzed test-substrate *p*-nitrofenil palmitat (*p*-NPP) as 1 µmol *p*-nitrofenol (product) per minute.

Measurement of emulsification activity

Emulsification activity was measured by visual method. Biosurfactant solution or crude enzyme as much as 1 mL was put on reaction tube and added 1 mL of kerosene as hydrocarbon substrate tested. This mixture was vortexed with high speed for 2 minutes and observed percentage of emulsification after 1 hour (AE 1h) and 24 hours (AE 24h). Emulsification activity value obtained from percentage of emulsion high that formed divided by total of liquid high [16]. Emulsification activity value was represented in % and reported as an average result of triplicate replications.

Measurement of surface tension

Surface tension of biosurfactant solution or crude enzyme was measured using Du-Nouy tensiometer. Value of surface tension represented as mN/m and reported as an average result of triplicate replications.

Oil sludge solubility test

Variation treatments in this research contain of eight treatments with triplicate replications (Table 1). Those treatments were vortexed at 30^{o}C for 15 minutes. Dissolved oil sludge (in liquid phase) was separated from oil sludge in a solid phase for the first by taking it using a pipette carefully. Then, centrifugation was done at 9000 rpm, 4°C for 15 minutes.

Table 1. Variation of treatments for oil sludge solubility test

Treatment	Oil sludge (g)	Biosurfactant of *Bacillus subtilis* 3KP [=CMC] (mL)	Tween-20 [=CMC] (mL)	Type of crude enzyme	
				Lipase (mL)	Protease (mL)
Tween-20	0.2	0	6	0	0
Biosurfactant	0.2	6	0	0	0
Protease	0.2	0	0	0	6
Lipase	0.2	0	0	6	0
Lipase + protease	0.2	0	0	3	3
Biosurfactant+lipase	0.2	3	0	3	0
Biosurfactant+protease	0.2	3	0	0	2
Biosurfactant+lipase+protease	0.2	2	0	2	2

Liquid phase filtration of each treatment

Whatman No. 1 filter paper with pore size 11µm and lipid-free funnel were prepared for liquid phase filtration of each treatment. Filter paper was used for filtration wrapped with aluminium foil which was weighed with analytical balance and wrote the weights W_o. Sample of liquid phase of centrifugation product as much as 1 mL was poured in the lipid-free funnel which put a filter paper inside. Oil sludge solubility was measured from separated and trapped oil on that filter paper. After filter paper leaked through and there was not liquid drop, filter paper was dried on oven at temperature 51-55 °C for 28 minutes. Dried filter paper was weighed again and wrote its weight as W_t. There was always a control (blank) in every treatment in oil sludge solubility test. Control (blank) was biosurfactant solution weight or crude enzyme without oil sludge addition and it treated the same as other. Weight of control (blank) of each treatment wrote as W_b.

Extrapolation of oil sludge solubility percentage

Dissolved oil sludge weight was counted using this formula:

$$W_{ot} = (W_t - W_o) \text{ x Total volume}$$

W_o, W_t, and W_{ot} are filter paper weight (g), weight of filter paper and dissolved oil sludge (g/mL), and weight of dissolved oil sludge (g/total volume) respectively.

Percentage of oil sludge solubility of each treatment can be counted with this formula:

$$\% \text{ oil sludge solubility} = \frac{W_{ot} - W_b}{W_{op}} \times 100\%$$

W_{ot}, W_b, dan W_{op} are weight of dissolved oil sludge (g), weight of control treatment (blank) (g), and weight of oil sludge treatment (g) respectively.

Statistical Analysis

Data of oil sludge solubility (%) was analyzed statistically using *One-way Analysis of Varians* (ANOVA) test (signification degree 5%) on *Statistical Package For Social Science* (SPSS) program. Is was conducted to know the presence of different signification inter group of given treatments and continued with Duncan test to know a pair of treatment groups which has a different oil sludge solubility value significantly.

RESULTS AND DISCUSSION

The characteristic of *Bacillus subtilis* 3KP biosurfactant

Biosurfactant produced by *Bacillus subtilis* 3KP on molasses substrate was included in bioemulsifier groups, and having a similarity to surfactin, a commercial biosurfactant [11]. Surfactin produced by *Bacillus subtilis* ATCC 21332 is one of the most effective biosurfactant. At lowest concentrations (0.005 %), surfactin can decrease water surface tension, from 72.0 to 27.9 mN /m [3]. Biosurfactant used in this study is a crude product obtained by 60% saturation ammonium sulphate precipitation. This biosurfactant have an equal concentration value with CMC (*Critical Micelle Concentration*) (± 16000 mg/L) [11]. CMC value of surfactant depends on the structure of surfactant, pH, ionic strength, temperature of solution, and solvent polarity that used to dissolve surfactant [15]. Characteristic of *Bacillus subtilis* 3KP biosurfactant at the CMC was shown in Table 2.

Table 2 Characteristic of *Bacillus subtilis* 3KP biosurfactant and synthetic surfactant of Tween-20 in each CMC value

Type of surfactant	Emulsification activity in kerosene (%)		Surface tension (mN/m)	Surface tension reduction (mN/m)	
	1 hour	24 hours		Compared with aquadest	Compared with solvent[a]
***B. subtilis* 3KP biosurfactant**	59.04 ± 0.02	56.93 ± 0.05	44.54 ± 1.87	27.46 ± 2.16	34.53 ± 7.55
Tween-20	49.10 ± 6.82	45.97 ± 6.38	43.54 ± 0.53	28.46 ± 0.53	32.79 ± 0.53

[a] Buffer Phospat pH 7 was used as solvent

The characteristic of crude protease and lipase

The results of semi-quantitative of crude protease from *Bacillus* sp. RPH 2.3 and crude lipase from *Actinobacillus* sp. P3-7 were shown on Table 3. The crude enzymes were also tested its emulsification activity and surface tension to detect the presents of surface-active compound (surfactant). A surface-active agent has an ability to form micro emulsion and lowering the surface tension [3]. The crude protease has high emulsification activity but less potential in decreasing surface tension. These indicated that crude protease from *Bacillus* sp. RPH 2.3 able to acts like an emulsifier. Meanwhile, crude lipase has no emulsification activity but less potential in decreasing surface tension.

Table 3 Characteristic of crude lipase and protease

Type of crude enzyme	Hallo zone diameter (mm) (after 24 hours)	Enzyme activity in test substrate*	Emulsification activity towards kerosene (%)		Surface tension of crude enzyme (mN/m)
			1 jam	24 jam	
Lipase	0.31 ± 0.04	0.07[a] U/mL	4.91 ± 3.19	0.00 ± 0.00	52.09 ± 0.04
Protease	15.03 ± 3.86	29.74[b] U/mL	63.67 ± 4,32	60.09 ± 1.11	59.34 ± 0.95

[a] *p*-nitrofenil palmitat (*p*-NPP) was a substrate for lipase assay
[b] Casein was a substrate for protease assay
*Activity of lipase and protease enzymes were measured at pH 7 and temperature 37°C

The percentage of oil sludge solubility

The variation treatments by adding *Bacillus subtilis* 3KP biosurfactant, crude lipase from *Actinobacillus* sp. P3-7, protease from *Bacillus* sp. RPH 2.3 and the combination of them were tested its ability in dissolving oil sludge (Figure 1). Aquadest was also tested its ability in dissolving oil sludge. Aquadest has no ability in dissolving oil sludge (0%).

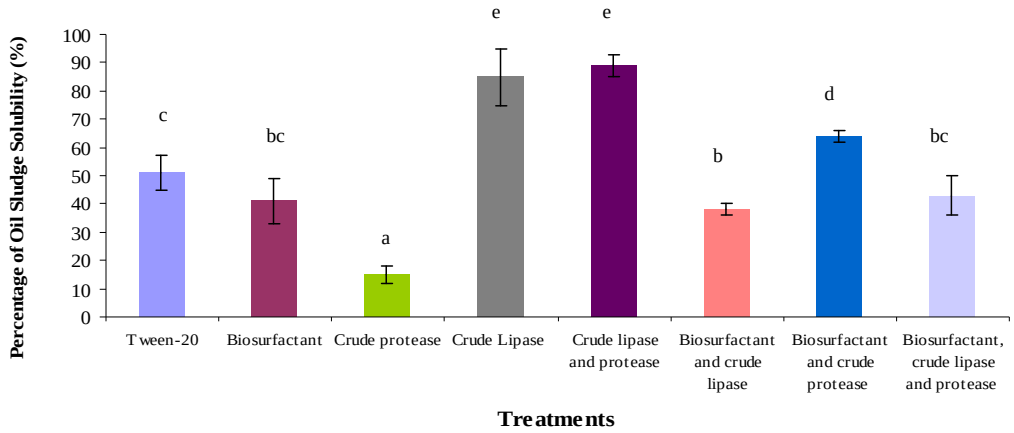

Figure 1. Percentage of oil sludge solubility on each treatment. Error bars show a deviation standard of three duets on each treatment. Different notation based on Duncan test.

Based on statistics test using one-way Anova, it can be known that variations of all treatments influencing solubility of oil sludge. Further results analysis using Duncan (p= 0.05) indicated that there are pairs groups of treatment with a significant different on oil sludge solubility (Figure 1).

In group treatment by surfactant addition, the solubility of oil sludge of biosurfactant (41%) was lower than using Tween-20 (51%). Tween-20 was used as representatives from synthetic surfactant. Characteristic of Tween-20 was described on a Table 2. But statistically, solubility of oil sludge by *Bacillus subtilis* 3KP biosurfactant and Tween-20 are not significant different. Biosurfactant could be equally effective with synthetic surfactant [14].

In the treatments with crude enzymes, combination of crude lipase from *Actinobacillus* sp. P3-7 and protease from *Bacillus sp*. RPH 2.3 (1: 1 v/v) produced a highest percentage of oil solubility (89%). However, statistically, oil sludge solubility of crude lipase and protease was not significant different with only crude lipase (85%). Crude protease produced lowest oil sludge solubility (15%). The largest content of oil sludge was petroleum hydrocarbon, such as oil or lipid [2]. Lipase will degrade oil or lipid which not soluble on water became soluble products [17] and catalyses lipid hydrolysis reaction on interfacial oil and water [5]. Lipase enzyme from *Actinobacillus* sp. P3-7 act on interfacial of lipid and water. And, it also degrade well lipid compounds in oil sludge, so that it can dissolve in water phase and yielding high percentage of oil sludge solubility.

Combination of *Bacillus subtilis* 3KP biosurfactant and crude enzyme was expected produce oil sludge solubility higher compared to a single use of *Bacillus subtilis* 3KP biosurfactan or crude enzyme. However, in fact, combination of *Bacillus subtilis* 3KP biosurfactant and crude enzyme oil resulted different in its solubility response. The solubility of oil sludge by combination of *Bacillus subtilis* 3KP biosurfactant and crude protease from *Bacillus sp.* RPH 2.3 (64%) is higher compared to single treatment of biosurfactant (41%) and crude protease (15%). Statistically, the solubility of oil sludge by combination of biosurfactant and crude protease produced a significant difference with biosurfactant and crude protease. While, the solubility of oil sludge by combination of biosurfactant and crude lipase (38%) produces lower yield compare to single treatment biosurfactant (41%) and lipase (85%). Statistically, the solubility of oil sludge by combination of biosurfactant and crude lipase was significantly different with crude lipase but was not significantly different with biosurfactant. Meanwhile, the combination of *Bacillus subtilis* 3KP biosurfactant with crude lipase from *Actinobacillus* sp. P3-7 and crude protease from *Bacillus* sp. RPH isolate 2.3 (1: 1: 1) (v/ v/ v) produced higher oil sludge solubility value (43 %) compared with biosurfactant treatment (41 %) and P (15 %). But, those result lower than crude lipase (85%).

This result indicates that interaction between *Bacillus subtilis* 3KP biosurfactant with crude protease and crude lipase gave different response or influence against oil sludge solubility. It was assumed that there was a positive interaction between *Bacillus subtilis* 3KP biosurfactant with crude protease from *Bacillus* sp. RPH 2.3. Interaction positive responses like this also happened in research conducted by [17] that mixing supernatant of *Bacillus subtilis* IAM 1213 culture containing biosurfactant with supernatant of *Aeromonas sp.* NKB26 culture containing enzymes such as lipase or protease, called as biocleaner. This combination produces higher efficiency in cleaning of crude oil from factory buildings, ceramic tile compared to a single use of *Bacillus subtilis* IAM 1213 or only *Aeromonas sp.* NKB26c. This research illustrating that combination of biosurfactant

and enzymes such as lipase or protease may increases the activity of oil cleaning. Hydrophilic and hydrophobic molecules of biosurfactant will cause oil and protein changes to be soluble in water. Protease or lipase enzyme will degrade oil and protein which is not soluble in water changing into products which is dissolves in water.

While, in combination of biosurfactant and crude lipase, it was assumed that there was a negative interaction between *Bacillus subtilis* 3KP biosurfactant with crude lipase from *Actinobacillus* sp. P3-7. This negative response is also found in research conducted by [17] on combination of *Bacillus subtilis* IAM1213 supernatant containing biosurfactant with supernatant of *Bacillus cereus* NKB46b culture containing enzymes. This condition caused by an inhibitory activity of lipase enzyme by biosurfactant. Biosurfactant is product of microorganisms that having similar properties to surfactant [18]. The presence of surfactant in reaction media of lipase enzymes catalysis can affect its hydrolysis level, it relates to the formation of enzyme-surfactant complex. Its catalytic property can differ with original enzymes.

In some cases, surfactant includes in a group of non-competitive inhibitor from lipase enzyme [7]. Non-competitive inhibitor defined as a compound indirectly act on the active site, but inhibits lipase activity by changing lipase conformation or interfacial characteristic (interface) [5]. The fact indicates surfactant influence more pertaining to interaction with enzymes on oil-water interfacial than on providing larger interfacial surface. *Bacillus subtilis* 3KP biosurfactant was assumed to be non-competitive inhibitor from lipase enzyme of *Actinobacillus* sp. P3-7. That based on the fact that lipase enzyme catalyzes lipid hydrolysis reaction on interfacial lipid-water [5]. Meanwhile, biosurfactant which was amphipatic molecule could be on liquid interfacial of liquid that have polar characteristic and different hydrogen bond, like oil-water [3]. Biosurfactant is also able to improve interfacial region [19]. Interfacial characteristic changing by surfactant existence can be the cause of lipase activity declines, where surfactant adsorption tested on interfacial (interface) oil-water can prevent or delay lipase penetration on interfacial [9]. So, the presence of *Bacillus subtilis* 3KP biosurfactant on interfacial oil-water prevents penetration of lipase enzyme of *Actinobacillus* sp. P3-7 on interfacial, so solubility of oil sludge is being reduced. These conditions occur because of the possible reaction products accumulation (mostly digliseride and free fatty acids) on interfacial, which will increase the speed of enzyme reaction declines [8].

CONCLUSION

The variations of *Bacillus subtilis* 3KP biosurfactant, crude enzymes, and the combination of both have impacted on percentage of oil sludge solubility. The combination of crude lipase from *Actinobacillus* sp. P3-7 and crude protease from *Bacillus* sp. RPH 2.3 gave the highest value of oil sludge solubility (89 ± 4,5%).

ACKNOWLEDGEMENT

This research was supported by DP2M-DIKTI of Republic of Indonesia (Project Number: 2613/H3/KR/2012, March 9th , 2012 through the foundation for Penelitian Unggulan Perguruan Tinggi (UPT), Universitas Airlangga, 2012.

REFERENCES

1. Banat, I.M. and I. Rancich. 2009 No man Entry Tank Cleaning and VOC Control Using Biotechnological Interventions. Environmental Technologies for Refineries programme. University of Ulster. Europe.
2. Budihardjo, M.A. 2007 Studi Pengaruh Bulking Agents pada Proses Bioremediasi Lumpur Minyak. Jurnal Purifikasi 8, 55-60.
3. Desai, J.D. and I.M. Banat. 1997 Microbial Production of Surfactant and Commercial Potential. Microbiol and Molecular Rev. 61, 47-64.
4. Fiechter, A. 1992. Biosurfactants: Moving Towards Industrial Application. Trends in Biotechnology 10, 208–217.
5. Gupta, R., N. Gupta, and P. Rathi. 2004 Bacterial Lipase: An Overview of Production, Purification and Biochemical Properties. Appl Microbiol Biotechnol. 64, 763-781.
6. Hou, C.T. and T.M. Johnston. 1992. Screening of Lipase Activities with Culture from The Agricultural Research Culture Collection. J. Am. Oil. Chem. Soc. 69, 1088-1097.
7. Iizumi, T., K. Nakamura, and T. Fukase. 1990 Purification and Characterization of a Thermostable Lipase from Newly Isolated *Pseudomonas* sp. KWI-56. Agric. Biol.Chem. 545, 1253-1258.

8. Jurado E., F. Camacho, G. Luzo´n, M. Ferna´ndez-Serrano, and M. Garcı´a-Roma´n. 2006.Kinetic Model for The Enzymatic Hydrolysis of Tributyrin in O/W Emulsions. J. Chem Eng Sci. 61, 5010-5020.

9. Jurado, E., V. Brav, G. Luzo, M. Fernandez-Serrano, M. Garcia-Roman, D. Altmajer-Vaz, and J.M. Vicaria. 2007 Hard-Surface Cleaning Using Lipases: Enzyme-Surfactant Interactions and Washing Tests. J. Surfact Detergent 10, 61-70.

10. Ni'matuzahroh, T. Surtiningsih, and Isnaeni. 2003. Kemampuan Bakteri Hidrokarbonoklastik dari Lingkungan Tercemar Minyak dalam Memproduksi Biosurfaktan: Upaya Bioremediasi Lingkungan. Laporan Penelitian RUT VIII.3. Lembaga Penelitian Universitas Airlangga. Surabaya.

11. Ni'matuzahroh, Fatimah, and T. Nurhariyati. 2010 Kinetika Produksi Biosurfaktan *Bacillus subtilis* 3KP Pada Molase. Laporan Penelitian Fundamental Tahun Anggaran 2010. Universitas Airlangga.

12. Pereira-Meirelles, F.V., M.H.M. Rocha-Leão, and G.L. Sant'Anna Jr. 1997 A Stable Lipase from *Candida lipolytica*: Cultivation Conditions and Crude Enzyme Characteristics. Appl. Biochem. Biotechnol 63-65, 73-85.

13. Pruthi, V., and S.S. Cameotra. 1997 Rapid Identification of Biosurfactant Producing Bacterial Strain Using a Cell Surface Hidrophobicity Techniques. Biotechnol Technique 11, 671-674.

14. Singh, A., J.D. Van Hamme, and O.P. Ward. 2006 Surfactants in Microbiology and Biotechnology: Part 2. application aspects. Biotechnology Advances.

15. Soberón-Chávez and Gloria. 2010 Biosurfactants: From Genes to Applications. Springer Microbiology Monographs 20, 2-4.

16. Tabatabaee, A., M. Mazaheri Assadi, A. A. Noohi, and V. A. Sajadian. 2005 Isolation of Biosurfactant Producing Bacteria from Oil Reservoirs. Iranian J Env Health Sci Eng. 2(1), 6-12.

17. Takeyama, H., M. Wada, and T. Matsunaga. 2002 Screening of Soil Bacteria for Production of Biocleaner. J. Applied Biochemistry and Biotechnology 2, 98-100.

18. Thavasi R, M.S. Nambaru , S. Jayalakhsmi, T. Balasubramanian, and I.M. Banat. 2009 Biosurfactant Production by *Azotobacter chroococcum* Isolated from the Marine Environment. Mar Biotechnol 11, 551-556.

19. Vater, J.,B. Kablitz,C.Wilde,P. Franke, N. Mehta,and S.S Cameotra. 2002. Matrix-assisted Laser Desorption Ionization–time of Flight Mass Spectrometry of Lipopeptide Biosurfactants in Whole Cells and Culture Filtrates of *Bacillus subtilis* C-1 Isolated from Petroleum Sludge. *Appl Environ Microbiol* 68, 6210–6219.