Prospective Study of *Bacillus sphaericus* and *Pseudomonas aeruginosa* as The Microbial Enhanced Oil Recovery Agents

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Abstract. Microbial Enhanced Oil Recovery (MEOR) is a microbial-based technology to obtain the maximum oil gaining in the oil reservoirs. The adapted bacterial as MEOR agents should be able to produce biosurfactant, high emulsification index values (E_{24}) and capable of lowering the surface-tension in crude oil. The present study undertakes the applicability of two bacterial species *Bacillus sphaericus* and *Pseudomonas aeruginosa* as MEOR agents. Both isolate as indigenous bacteria from West Java oil sludge. The aim of this study is to obtain the best biosurfactant-producing microbe with the highest emulsification index value and the lowest surface-tension in crude oil. Our result shows that *P. aeruginosa* is more suitable than *B. sphaericus* to be used as MEOR agent with the highest emulsification index values (by 65%). This microbe is also able to decrease the surface-tension of crude oil by 7 dyne cm^{-1}. Concurrently, *B. sphaericus* obtains 55% of emulsification index values and is capable of decreasing the surface-tension of crude oil by 2 dyne/cm. *P. aeruginosa* is more suitable than *B. sphaericus* to be used as MEOR agent

1. Introduction

Recently, the annual *crude oil* demand in Indonesia disproportionally increases with its production by 1.7%. In addition, this *crude oil* production has decreased by 1.2% over the last two years. This problem occurs due to the inefficient technique of oil withdrawal that can only obtain 40% of crude oil. Moreover, the remaining crude oil attached to the reservoir can be acquired by tertiary technique using either surfactant or biosurfactant [1]. Due to environmental concern, the demand for biodegradable surfactants is increasing. Moreover, utilization of bio-compounds such as biosurfactant as an alternative to conventional surfactants has been urged due to the rapid development in biotechnology and its advantages, such as lower toxicity and higher biodegradability.

Biosurfactants or surface-active agents are amphiphilic molecules that comprise both hydrophobic and hydrophilic moieties, being the apolar component usually a carbon chain, whereas the polar part, more variable, can be ionic (anionic or cationic) or non-ionic. Those compounds are able to reduce surface and interfacial tensions, as well as to form and stabilize oil in water or water in oil emulsions. Among several potential applications of biosurfactants, its use in MEOR represents one of the most promising methods to recover substantial amounts of the residual oil entrapped in mature oil fields [2]. Indeed, the biosurfactant is able to reduce the surface-tension of oil and water which is a crucial parameter in the Microbial Enhanced Oil Recovery (MEOR) [3].

Oil cannot mix with water due to its hydrophobic characteristic. This occurs due to the difference in interfacial tension between oil and water. Therefore, the interfacial tension of the oil should be

lowered to be soluble in water by adding the biosurfactant.

Over the last years, several biosurfactant-producer microorganisms have been used such as *Bacillus subtilis*, *Bacillus sphaericus* and *Pseudomonas aeruginosa* [4]. In this research both bacteria is indigenous microorganism from west java oil sludge. Several characteristics should be met by the MEOR agent, for instance, the ability of producing biosurfactant, high emulsification index values and decrease the interfacial tension of the crude oil. The present study aims to compare the production of biosurfactants produced by *Bacillus sphaericus* and *Pseudomonas aeruginosa* as the MEOR agents.

2. Materials and Methods

2.1. Determination of biosurfactant production

The ability of microorganisms in producing biosurfactant was tested using the blood agar haemolytic method. Briefly, the haemolytic activity of the bacterial strain was observed by inoculation of bacteria on the blood agar medium with the horizontal streak. The inoculum was further incubated at 37 ^{o}C in 24 h. The biosurfactant producer bacteria were observed by determining the transparent zone formed around the bacterial colonies [5].

2.2. Determination of microbial growth curve

Bacterial cell growth was monitored by measuring the dry cell weight method. It was determined by centrifugation (10,000 rpm for 30 minutes) of a 1 ml culture broth; the cell pellet was washed with distilled water twice and dried by heating at 50 ^{o}C until constant weight was attained.

2.3. Emulsifying activity determination (E^{24})

The emulsifying activity was conducted to determine the emulsification level of total fraction of the biosurfactant against the crude oil. The measurements were made by adding 5 ml of centrifuged supernatant to 5 ml of crude oil. The inoculum was further incubated at 37 ^{o}C in 24 h. Afterwards, the mixed solution between the crude oil and the supernatant was vortexed with maximum speed until perfectly mixed, and then it was incubated for 24 h. The Emulsification Index (E^{24}) was finally determined by measuring the height of the emulsified oil solution divided by the total solution after 24 h [7].

2.4. Surface-activity determination

The surface-activity was measured by using the Du-Nuoy method. The measurement was performed in order to determine the surface-tension of the crude oil in the bacterial growth medium. Briefly, the microbe age 5 days Macfarland 3 was inoculated into 100 ml of Salt Medium SS medium in 150 ml glass bottle. After, a total of 10% of crude oil was added into the medium. Moreover, the surface-tension was measured prior to incubation and then re-measured after 7 days of incubation with the Du Noy tensiometer.

2.5. Data analysis

All of the data obtained from the experiment were descriptively analysed. Additionally, general description of the data was obtained by measuring the average, the minimum and the maximum value.

3. Result and Discussion

3.1. Determination of biosurfactant production

Blood haemolysis method is commonly used to detect the presence of biosurfactants produced by the microbes, i.e. indicated by the formation of haemolysis (lysis zone) around the colony. The blood agar haemolysis method was carried out using potential biosurfactant of the lipopeptide antibiotic group causing the lysis of erythrocyte cells and clotting inhibitors as reference [8]. This was because the surfactant serves as a haemolysin substance. Haemolysin could act as an antibody to the erythrocyte

membrane antigen, which makes it undergo haemolysis [9]. Our result showed that both *P. aeruginosa* and *B. sphaericus* were able to haemolysed the blood agar.

Table 1. Tests on haemolysis of blood agar

Species name	Transparent zone (cm)	Haemolysis	
		Type	Color
Pseudomonas aeruginosa	0.55	B	transparent
Bacillus sphaericus	0.3	B	transparent

The isolate of *P. aeruginosa* was able to haemolyse the blood agar with the clear zone as much as 0.55 cm (Table 1). On the other hand, *B. sphaericus* isolate produced the clear zone as much as 0.3 cm. Based on clear zone formed, it appears that *P. aeruginosa* has the ability to haemolyse the blood agar more than *B. sphaericus*.

The haemolysis that occurs in both isolates was categorized as beta haemolysis (β) due to its ability to haemolyse the entire red blood cells in the media, thus the colour around the colony became clear (Figure 1).

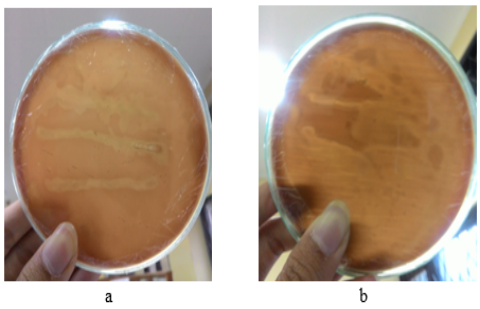

Figure 1. The clear zone formed in haemolysis of *P. aeruginosa* (a) and *B. sphaericus* (b) isolates

Our findings were coherence with the study from Daud et al. [10], who also observed the bacteria that experienced β-haemolysis are the most potential bacteria to produce biosurfactants. In addition, this finding was also confirmed by Ni'matuzahroh [11] who isolated *Pseudomonas* sp. with β-haemolysis type from the coastal area of Surabaya. In contrast, the bacterial isolate without haemolytic activity did not produce biosurfactant [12].

3.2. Bacterial growth curve

In the present study, the initial colony number of *P. aeruginosa* was as much as 1.39 x 10^{12} CFU ml^{-1}. This number was exponentially increased on the third day into 3.90 x 10^{12} CFU ml^{-1}. The optimum number of colonies occurred on day 9 as much as 4.45 x 10^{12} CFU ml^{-1} (Figure 2).

In this study, the exponential phase appeared from day 0 to day 9, indicated by the significant increase of the bacterial colony number. This phase occurs when the bacterial cells utilized the existing nutrient, to the extent of their increase. Moreover, in this phase there is an increase in the number of cleavage and the formation of cell constituents in order to produce an increasing number of cells. In addition, the primary metabolite was also formed in this phase, which is the material of cell constituents and components that increase the amount of metabolism [13].

P. aeruginosa is known as biosurfactant producer in form of rhamnolipid. Indeed, this biosurfactant is an active compound produced by the bacteria that is able to increase the surface tension [14].

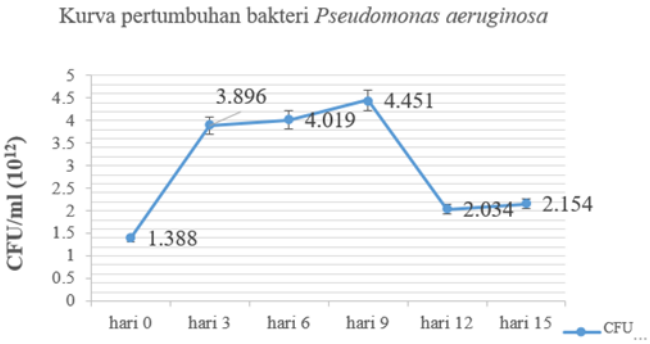

Figure 2. Growth curve of *P. aeruginosa*

In our study, the exponential phase occurred on the day 3, where the number of bacterial cells increased 3 times more than their initial number. However, the division rate on the 6th and 9th days only ranged from 1.03 to 1.11 times from the number of bacterial cells of the previous observation.

Our results revealed that the adaptation phase in *P. aeruginosa* occurred from day 3 to day 6, which subsequently re-increased on day 9. However, observation on day 12 revealed that the bacterial colony increased from 4.45 x 10^{12} to 2.03 x 10^{12} CFU ml^{-1}.

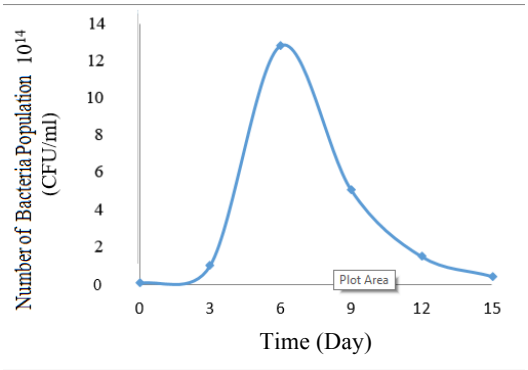

Figure 3. Growth curve of *B. sphaericus*

Growth curve of *B. sphaericus* demonstrated that the exponential phase occurred from day 0 to day 6 (**Figure 3**), where the bacterial population significantly increased. In this phase, the number of bacteria increased from 10.80 x 10^{12} (on day 3) to 106.83 x 10^{12} CFU g^{-1}. Moreover, at the end of exponential phase (day 6), this number increased to 1280 x 10^{12} CFU g^{-1}.

The decline of bacterial population occurred right after day 6 until day 15. The number of bacteria decreased at day 9 from 1,280 x 10^{12} to 511.60 x 10^{12} CFU g^{-1}. Furthermore, the decline on the bacterial population continued from 511.60 x 10^{12} to 155 x 10^{12} CFU g^{-1} (day 12) and further decreased to 49 x 10^{12} CFU g^{-1} (day 15).

To our knowledge, the decline in bacterial population after the exponential phase is relatively common in batch fermentation system due to limiting factors that will be achieved during fermentation. These limiting factors are the decrease of nutrients sources and the accumulated auto-toxic products released by bacteria into the fermentation medium [13].

3.3. Emulsification Index (E_{24})

The emulsification index can be obtained by measuring the height of the emulsified oil solution divided by the height of the total solution [7]. Based on the emulsification index value, we demonstrated that *P. aeruginosa* had better ability to emulsify crude oil than *B. sphaericus* (Table 2).

Table 2. Emulsification index of *P. aeruginosa* and *B. sphaericus*

Species	Emulsification Index value (%)
P. aeruginosa	65
B. sphaericus	55

The emulsification index value of *P. aeruginosa* was 65%, while that of *B. sphaericus* was 55%. Indeed, emulsification capability is one of the requirements of microorganism to be an ideal candidate as MEOR agent. Emulsification could be defined as a process when the two fluids cannot be mixed, thus it needs to be stabilized by the emulsification agent, such as biosurfactant, to form an emulsion. This emulsification capability can be used to increase the oil production by emulsifying the trapped crude oil in the rock thus it can be easily removed with water.

The emulsification index states the precentage of capability of emulsion formation by bacterial isolate. Furthermore, this index can be used to select isolates of biosurfactant-producing bacteria. The large percentage of stable emulsions formed indicate the greater amount of biosurfactant produced by the isolates [15]. The applicability of biosurfactants in several fields depends on their stability at different temperatures. The stability of biosurfactant was tested over a wide range of temperatures. The biosurfactant produced by *Bacillus* and *Pseudomonas* species respectively was shown to be thermo stable as indicated by the stability in the surface tension values and the oil displacement area. Therefore, it can be concluded that these biosurfactants maintains their surface properties unaffected in the range of temperatures between 30 and 100^{o}C.

3.4. Surface tension

The surface tension could be defined as the effort required to expand the liquid surface per unit area. Indeed, the surface tension is a force that arises along the surface line of a liquid. This force arises due to the contact between the two different fluids phase. In our study, the surface tension measurement was performed at the initial and at the end of incubation (Table 3).

Tabel 3. Surface tension on *P. aeruginosa* and *B. sphaericus*

Species	Surface tension	
	Before incubation (dyne cm^{-1})	After incubation (dyne cm^{-1})
P. aeruginosa	27	20
B. sphaericus	27	25

Our results showed that both bacterial species were able to decrease the surface tension of crude oil after incubation. The surface tension for both species prior to incubation was similar 27 dyne cm^{-1}. Afterwards, the surface tension in both bacterial species decreased after 7 days of incubation (20 and 25 dyne cm^{-1} for *P. aeruginosa* and *B. sphaericus*, respectively). In other words, *P. aeruginosa* isolate was able to decrease surface tension as much as 7 dyne cm^{-1}, while *B. sphaericus* isolate was only able to decrease as much as 2 dyne cm^{-1}. Therefore, *P. aeruginosa* had a better ability in lowering the surface tension than *B. sphaericus*.

The effect of sodium chloride addition on biosurfactant produced from *Bacillus* and *Pseudomonas* species was studied. the stability of biosurfactant produced by *Pseudomonas aeruginosa* at NaCl concentration range of 2-20% (w/v) and biosurfactant produced by *Streptomyces sp*. at NaCl concentration range of 1-9% (w/v), respectively.Diminution in surface tension occurs due to the exudates of extracellular compounds by the bacteria with surface active properties (biosurfactant) during the incubation period. Biosurfactant is composed by the hydrophobic and hydrophilic groups on its molecule. It tends to be appeared at the interface between the two different phases of polarity [16]. Biosurfactant is equally able to decrease the surface tension between the two phases. Different

polarity properties between the two phases of liquids affect their low solubility. Hence, the presence of surfactant molecules that have a tendency towards both phases, the two liquids can be mixed together.

The fluid molecules that present on the surface undergo the resultant force toward the fluid body. Therefore, these molecules tend to squeeze inward to avoid the surface, where the molecules in the fluid have a balanced resultant force. The tendency of particles to move into the body fluids produce a force, and the amount of power required to break the surface of the liquid thus forming a new extent on the surface, called the surface tension [17].

Surface tension is negatively correlated to emulsification index. Thus, the higher emulsification index results to the lower surface tension. The microorganism as MEOR agent should be able to reduce the surface tension of crude oil. Thus, a low surface tension of crude oil will facilitate its removal from the reservoir. Indeed, low surface tension makes crude oil emulsify with water easily. Therefore, the crude oil will be mixed with water and easier to remove from the reservoir.

The present study demonstrates that *P. aeruginosa* isolate has not only better emulsification ability, but also in reducing the surface tension than that of *B. sphaericus*. To our knowledge, genus *Pseudomonas* has been widely recognized as one of the microbial groups that have favourable ability in degrading crude oil. This genus also has the ability to degrade aliphatic, aromatic and resin fractions. Furthermore, genus *Pseudomonas* can amplify better by using crude oil as its carbon source [11].

4. Conclusion

The present study demonstrates that *P. aeruginosa* has higher emulsification index than *B. sphaericus*. The observed emulsification index of *P. aeruginosa* is as much as 65%, which is higher than that of *B. sphaericus* (55%).

Our findings also observed that both species are able to reduce the surface tension of crude oil in the medium of bacteria. The surface tension decreases as much as 7 and 2 dyne cm^{-1} for *P. aeruginosa* and *B. sphaericus*, respectively. Further research is required to determine an ideal media to produce biosurfactant from both bacterial species.

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5. References

[1] Agarry S E, Salam K K, Arinkoola A and Aremu M O 2015 Biosurfactant production by indigeneous *Pseudomonas* and *Bacillus* species isolated from auto-mechanic soil environment towards microbial enhanced oil recovery *European journal of engineering Technology* **3** ISSN 2056 5860.

[2] Rossiana N and Wulandari A P 2015 Biosurfactants activity from exogenous fungi to decreasing total petroleum hydrocarbon (tph) of balongan oil sludge *Asian Jr. of Microbiol. Biotech. Env. Sc*. **17** 781-784 ISSN-0972-3005.

[3] Fox S L 1993 Comperative analysis of microbially mediated oil recovery by surfactant produced by *Bacillus licheniformis* and *Bacillus subtilis International Conferences on Microbial Enhanced Oil Recovery.*

[4] Rossiana N, Kusmoro J, and Dhahiyat Y 2018 Utilization of *Talaromyces* sp., *Cladosporium* sp., and *Albizia* (*Paraserianthes falcataria* L. Nielsen) mycorhizae on the phytoremediation of oil sludge: change of lead, nickel total petroleum hydrocarbon (TPH) and polyaromatic hydrocarbon (PAH) *Journal of Petroleum and Environmental Biotechnology* **9** ISSN 2157 7463.

[5] Tabatabaee A, Assadi M M, Noohi A A and Sajadian V A 2005 Biosurfactant producing bacteria from oil reservoirs *Iranian J. Env. Health Sci. Eng*. **2** 6-12.
[6] Capucino J G and Sherman N 1987 *Microbiology: A Laboratory Manual*. The Benjamin Cumming Publishing Company, Inc.
[7] Suryatmana P, Edwan K, Enny R, and Wisnuprapto 2014 Karakteristik biosurfaktan dari *Azotobacter chroococcum*. *Laporan akhir dan Seminar Evaluasi RUT XI* 1-8. Serpong: Kementrian Riset dan Teknologi RI.
[8] Schulz D, Passeri A, and Schmidt M 1995 Screening for biosurfactants among crude-oil degrading marine microorganism from the North-sea *Z naturforch (C)* 197-203.
[9] Yatim W 1999 *Kamus Biologi* Yayasan Obor Indonesia.
[10] Daud M 2005 Performa ayam pedanging yang diberi probiotik dan prebiotik *Jurnal Ilmu Ternak* **5.**
[11] Ni'matuzahroh, Yachya A, and Tanjung M 2006 Studi perbandingan biosurfaktan *Pseudomonas aeruginosa* IA7 d dan surfaktan sintetik Tween-80 dalam *b*iodegradasi solar oleh mikroba perairan pelabuhan Tanjung Perak Surabaya P*enelitian Hayati* 13-18.
[12] Agustiani E D 1998 Penapisan bakteri penghasil biosurfaktan dari sumur minyak bumi *Skripsi* Institut Teknologi Bandung.
[13] Myers D 1946 *Sufactant science & technology*. Third Edition John Wiley & Sons.
[14] Martha M 2015 Low-income cancer survivors' use of health- promoting behaviors *Research for Practice: Medsurg Nursing* 50-62.
[15] Budijono and Hasby M 2010 Efektivitas pemakaian zeolit sebagai media biofilter limbah cair tahu *Jurnal Ilmu Perairan* 64-70.
[16] Vater J, Kablitz B, Wilde C, Franke P, Mehta N and Cameotra S 2002 Matrix assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactant in whole cell and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum slude *J. Appl. Environ. Microbiol*. 6210-6219.
[17] Nitschk M and Pastore G 2006 Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater *Bioresource Technology 336-341.*