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Potential Degradation of SARA (Saturated, Aromatics, Resinics, Asphaltenes) Fractions of Crude Oil by Reservoir Indigenous Bacteria from South Sumatera

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MEOR (*M*icrobial Enhanced Oil Recovery) technology utilizes metabolic activity of microorganisms such as degradation of hydrocarbon fractions which alters oil characteristics to facilitate and increase oil recovery from reservoir. This research focused on isolation of indigenous hydrocarbonoclastic bacteria that were capable of degrading SARA (Saturated, Aromatic, Resinic, Asphaltenes) fractions of crude oil to be utilized in MEOR. Sequential isolation of hydrocarbonoclastic bacteria were conducted using Nutrient Broth and Stone Mineral Salt Solution medium supplemented with 2% (v/v) crude oil and 0.1% (w/v) yeast extract and incubated at 50°C, 120 rpm agitation. Isolates retrieved were screened based on its activity to degrade crude oil, indicated by resazurin assay result. Physical and chemical characteristics of crude oil altered by selected isolates were observed using column chromatography, biometric test, GC-MS analysis, IFT, and viscosity measurements. Isolates with the best degradation activity were identified with 16S rRNA gene sequencing. Among thirty-one bacterial isolates obtained from sequential isolation, six isolates exhibited high oil degrading ability. SARA assay showed degradation activity of those isolates to each of SARA fraction were around 6-70%. This degradation was followed by significant CO_2 production ranging from 2000-4000 mg (value of $p<0.05$). Microbial degradation activity exhibited changes in chemical and physical characteristics of hydrocarbons showed by changes in composition of SARA fraction, decreased viscosity and IFT of crude oil 17-31%. This research identified three isolates with the best hydrocarbon fraction degrading ability were identified as different strain of *Bacillus licheniformis* and confirmed their high potential to be utilized in MEOR technology.

Key words: *Bacillus licheniformis, crude oil, GC-MS*, MEOR, SARA

Teknologi MEOR (*Microbial Enhanced Oil Recovery*) memanfaatkan aktivitas metabolisme mikroorganisme seperti degradasi fraksi hidrokarbon yang mengubah karakteristik minyak dan meningkatkan perolehan minyak dari reservoar. Penelitian ini difokuskan pada isolasi bakteri hidrokarbonoklastik yang dapat mendegradasi fraksi SARA (*Saturated, Aromatic, Resinic, Asphaltenes*) minyak bumi yang akan digunakan di MEOR. Bakteri hidrokarbonoklastik diisolasi secara bertahap dengan medium *Nutrient Broth* dan *Stone Mineral Salt Solution* ditambahkan dengan minyak bumi 2% (v/v) dan ekstrak ragi 0.1% (b/v), diinkubasi pada suhu 50°C dengan agitasi 120 rpm. Isolat yang diperoleh diskrining aktivitasnya dalam mendegradasi minyak bumi, ditunjukkan dari uji resazurin. Karakteristik fisik dan kimia minyak bumi yang berubah oleh isolat terpilih diamati dengan menggunakan kromatografi kolom, uji biometrik, pengukuran IFT, viskositas, dan analisis GC-MS. Isolat dengan aktivitas degradasi terbaik diidentifikasi melalui sekuensing gen 16S rRNA. Tiga puluh satu isolat bakteri diperoleh melalui isolasi bertahap dan enam isolat yang memiliki kemampuan degradasi minyak bumi tinggi. Uji SARA menunjukkan aktivitas degradasi isolat fraksi SARA minyak bumi sekitar 6-70% per fraksi. Degradasi diikuti oleh produksi CO_2 berkisar antara 2000-4000 mg secara signifikan (nilai $p<0,05$). Aktivitas degradasi mikroba menunjukkan terjadinya perubahan karakteristik kimia dan fisika hidrokarbon ditunjukkan melalui perubahan komposisi fraksi SARA minyak bumi dan penurunan viskositas dan IFT minyak turun sekitar 17-31%. Penelitian ini mengidentifikasi tiga isolat dengan kemampuan degradasi fraksi hidrokarbon terbaik, teridentifikasi sebagai strain *Bacillus licheniformis* yang berbeda dan terkonfirmasi memiliki potensi tinggi untuk digunakan dalam teknologi MEOR.

Kata kunci: *Bacillus licheniformis*, GC-MS, MEOR, minyak bumi, SARA

It is estimated that over two-thirds of crude oil in a mature reservoir remains untouched after primary and secondary recoveries and thus should be extracted using enhanced oil recovery (EOR) techniques (Safdel *et al.* 2017). Chemicals such as surfactants, emulsifiers, polymers, acids, and solvents have been used in tertiary recovery techniques to improve crude

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oil yield (Patel *et al.* 2015). However, chemical enhanced oil recovery methods are environmentally hazardous and expensive, and leave non-degradable residues because the chemical pathways through which these products are generated often use toxic chemicals (Lazar *et al*. 2007; Gudiña *et al*. 2012; Patel *et al.* 2015).

Microbial enhanced oil recovery (MEOR) is a low-priced technique with high potential in which different microorganisms and their metabolic products are exploited to recover the remaining trapped oil in reservoir (Lazar *et al*. 2007). MEOR is widely applicable in different types of reservoir such as sandstone and carbonate reservoirs with light/heavy crude oil and low/mid and high permeabilities (Long *et al.* 2013). The use of microbial cells, and microbial products such as biosurfactants, biofilms, biopolymers, and biologically-produced acids and solvents in MEOR have been shown to improve crude oil recovery. This method has advantages over traditional EOR techniques because these bioproducts can be produced with cheaper substrates, and are highly biodegradable and nontoxic (Lazar *et al*. 2007).

The main factors responsible for the poor oil recovery are low permeability of some reservoirs and inherent characteristics of heavy crude oil such as high viscosity, which limits its mobility, and high interfacial tension between hydrocarbon and aqueous phases, which results in high capillary forces that entraps oil in small pores within reservoir rock (Sen 2008; Brown 2010).

Nowadays, with the rising price of crude oil, declining reserves of medium and light crude oil and the abundance of unconventional crude oil (i.e. heavy oil), causing exploitation of heavy oil is favoured. Heavy oil are composed usually of viscous oils, high carbon-to-hydrogen atomic ratios, and display a greater content of asphaltenes and resins than conventional oils (Speight 1991), which directly impacts recovery, transport and refining processes. The rheological properties of heavy oils and the characteristics of their reservoirs make its production a challenge in the oil industry. In the past, heavy crude oil production was considered to be uneconomic, because of the intensive cost of recovery, transportation, refining and low market value (Santos *et al.* 2014).

Thermal methods are common in technologies used for the production of heavy oils (Shah *et al*. 2010). Thermal methods are based on supplying heat to the reservoir. In this way, the improvement in oil recovery is mainly due to the reduction of the oil viscosity and, consequently, to the improvement of the mobility ratio. The main processes that use thermal methods for heavy oil recovery are steam methods, like Cyclic Steam Stimulation (CSS), Steam Flooding (SF) and Steam Assisted Gravity Drainage (SAGD) (Santos *et al.* 2014). The major limitation of thermal method is it leaves considerable amounts of oil in the reservoir that can only be recovered by drive processes and it is observed that less than 30% (usually less than 20%) of the initial oil in place can be recovered. These steam methods are also damage the underground well structure and the equipment, endanger lives of the workers, expensive (i.e. add extra operational costs since larger volumes of liquids must be handled), and need an extra dehydration facility to allow for adequate separation of oil and water before oil shipment (Muggeridge *et al*. 2014). Therefore, the use of microbial to enhance oil recovery is an alternative way to produce high viscous crude oil with low-priced and environmental friendly. Microbial activity can improve sweep efficiency of heavy oil, thus improves oil recovery by lowering crude oil viscosity through degradation and increasing pressure due to gas production.

As demonstrated by previous studies, oil-degrading bacteria has provided many opportunities to improve oil recovery. The challenge is to find an appropriate isolates that can alter physical and chemical characteristics of crude oil, so that it suitable for use in MEOR technology. This research aimed to isolate indigenous hydrocarbonoclastic bacteria from in South Sumatera's well and further explored its ability to degrade petroleum using SARA (Saturated, Aromatic, Resinic, Asphaltenes) assay to determine its MEOR potential.

MATERIALS AND METHODS

Sample. Crude oil (oil characteristics: 22-28^{o} API) samples were taken from petroleum reservoir, South Sumatera.

Media. Stone Mineral Salt Solution (SMSS) was used to isolate potential oil-degrading bacteria. The composition of medium was as follows (g/L): NH_4NO_3 (2.5); $MgSO_4.7H_2O$ (0.5); $MnCl_2.4H2O$ (0.2); $CaCO_3$ (0.5); $Na_2HPO_4.7H_2O$ (1); KH_2PO_4 (0.5) (Sharpley 1966). The medium was supplemented with 2% (v/v) of crude oil (sterile) as the sole carbon source with the addition of 0.1% (w/v) yeast extract as nitrogen source. Bacterial growth was observed in Nutrient Agar (NA) Difco™ medium (Cappucino 2008).

Sequential Isolation of Oil-Degrading Bacteria. In the first stage of isolation, 2% (v/v) crude oil sample was inoculated into SMSS media enriched with 0,1% (w/v) yeast extract and 2% (v/v) sterilized crude oil. The culture was incubated at 50 °C with 120 rpm agitation for 7 d. Then, the culture was serially diluted to 10^7 CFU mL^{-1}, and plated into NA and SMSS agar using spread method. Plates of culture were incubated at 50 °C for 24-48 h. Emerging colonies were isolated and purified using four way streak method on NA and SMSS plates. In second stage of isolation, the carbon source was substituted with remaining-oil recovery degradated (ROD) from the first stage. Isolation of stage II was performed in the same way as the first stage. (Halim *et al.* 2008; Munawar *et al*. 2012).

Screening of Oil-Degrading Ability using Resazurin Assay. Screening of oil-degrading ability was carried out in two stages. In the first stage, bacterial isolates were initially cultivated in 10 mL of Nutrient Broth (NB) and incubated for 48 h at 50 °C with 120 rpm agitation. After incubation, the bacterial cell was centrifuged at 15,000 rpm for 10 min to obtain pellet. The pellet was washed with 5 mL of *Stone Mineral Salt Solution* (SMSS), centrifuged for another 10 min, and resuspended in 4 mL of SMSS medium. Resazurin assay was conducted by mixing 10 mL of SMSS medium, 1 mL sterilized crude oil, 1 mL of resazurin, and 50µL of bacteria isolate. Color change of resazurin from blue, pink to colorless indicate degradation rate. The isolates were incubated for 7 d in the incubator shaker at 50 °C and agitated 120 rpm. (Modified from Benedek *et al*. 2010).

Growth Curve. The growth of isolates with high oil degrading ability were studied using Total Plate Count. Sampling time was conducted with 12 h interval (Cappucino 2008).

Saturates, Aromatics, Resins and Asphaltenes (SARA) Separation of Crude Oil. Components of crude oil from each culture (before and after treatment) was separated using silica gel column chromatography (Muhammad *et al.* 2013). Total petroleum hydrocarbons (TPH) extract was dissolved in 10 mL of hexane and the insoluble fraction (asphaltene) was removed using Whatman filter paper and weighed. The soluble fraction was loaded on top of silica gel G (70-120 mesh) column (2 cm x 30 cm) and eluted with solvents of different polarities. The alkane fraction was eluted with 20 mL of hexane; aromatic fraction was eluted with 100 mL of toluene, and resinics fraction was eluted with 20 mL of methanol:toluene (90:10). All sample fractions were evaporated and weighed to calculate the weight of each fraction.

Biodegradation of Crude Oil In A Biometric System For Mineralization Test. Biometric flask was filled with 1 mL of a 48 h old activated inoculum, 28 mL of SMSS medium and 2% (v/v) hydrocarbon substrate. The sidearm of biometric flask was filled with 10 mL of 0.1 M KOH. Flasks were incubated at 50 °C with 120 rpm agitation. The culture was sampled every 24 h for 96 h from the sidearm of the flask and measured for its carbon dioxide content with colorimetric titration and gravimetric analysis. The control sample contained 10 mL of KOH, 1 mL of barium chloride and 0.1 mL of phenolphthalein. The amount of CO_2 produced was calculated using the equation below:

$$CO_2 \text{ generated(mg)} = (VB - VA) .MCO_2$$

$$CO_2 \text{ generated(mg)} = 2x \text{ MHClx CF}$$

VB = volume of HCl (0.1M) used to titrate the blank (mL)

VA = volume of HCl (0.1M) used to titrate the treatment (mL)

MCO2 = molar mass of carbon dioxide (g mol^{-1})

M HCl = molarity of HCl standard solution (mol L^{-1})

CF = correction factor for acid/base molarity (M HCl/M KOH).

All the experiments were conducted in triplicate and analyzed statistically (Modified from Benedek *et al*. 2010; Maier 2008; Biodia *et al.* 2010).

GC-MS Analysis of Residual Oil Components after Biodegradation. Liquid medium after biodegradation treatment was used as the sample, and non-inoculated medium without crude oil solution was used as a control. After 7 d of biodegradation, the medium was mixed with petroleum ether and centrifuged, the residual oil components for both the samples and the controls were measured by Agilent GC-MSD (6980N-5973) with temperature kept at 80 °C for 4 min, then increased at a rate of 5 °C·min^{-1} until 250 °C and maintained at 250 °C for 20 min.

Identification of Isolates using 16S rRNA Analysis. Selected hydrocarbonoclastic bacteria were sequenced using colony-based method. The sequencing used universal primer 785F (5'-CCAGCAGCCGCGGTAATACG-3') and 907R (5'-TACCAGGGTATCTAATCC-3') designed by Macrogen, Korea. The result was then analyzed using MEGA 6.0, to generate a phylogenetic tree with Neighbor Joining method (Tamura *et al.* 2011). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100

replicates). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. The analysis involved 27 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1355 positions in the final data set.

RESULTS

Sequential Isolation of Microorganisms and Screening of Oil-Degrading Ability using Resazurin Assay. Sequential isolation of hydrocarbon degrading bacteria obtained 12 isolates and 19 isolates from the first stage and second stage of isolation respectively, yielding a total of 31 isolates. Both Gram negative and Gram positive bacteria were observed among the isolates and the majority had rod-like shape. All isolates possessed the ability to degrade and utilize hydrocarbon, but six isolates namely DS1, DS3, DS19, DS31, DS32, and DS44 were found to exhibit higher efficiency of oil degradation as indicated by faster rate of resazurin color change.

Growth Rate and Crude-Oil Degrading of Selected Isolates. In general, logarithmic phase on 12 h to 48 h periods of incubation, stationary phase on 72 to 84 h and death phase at 96 h. were observed as the growth pattern of all isolates. Growth curve measurement was used to determine specific growth rate and the optimum time to use each bacteria as inoculum on further assays. The optimum growth rate was achieve at 12 h for isolates DS1, DS3, DS31 and DS32, and 24 h for isolate DS19 and DS44 (Fig 1).

Separation of SARA Components of Crude Oil Fractionated by Column Chromatography. SARA assay result is shown in the Fig 2. In general, all isolate degraded resin fraction into aromatics and saturated fractions. The highest degradation ability was observed on treatment by DS31 and followed by isolate DS44, DS3 and Ds1.

Biodegradation of Crude Oil in a Biometric System for Mineralization Test. Based on Fig. 3, most of the six tested isolates produced high amount of CO_2 gas after 96 h of incubation. The highest CO_2 production at 96 h incubation was shown by DS31 17000 mg. CO_2 production by DS1 7333 mg, DS3 11000 mg, DS19 8800 mg, DS32 7333 mg and DS44 5000 mg.

Crude Oil GC-MS Analysis. GC-MS analysis results (Fig 4) detected new compounds such as (3,5) - diosgenin, naphthalene, bicyclo (4.1.0) heptane, 1-methyladamantane, 1,6-dimethyl decalin, trans-1,6-dimethylspiro (4.5) decane, 2-isopropenyl-5-methyl-4-hexenyl acetate, cyclohexene, salvialane, decahydro-4,4,8,9, 10-pentamethylnaphthalene, cyclododecanone, fukinan, benzene, and azulene.

Interfacial Tension and Viscosity Reduction after Incubation Experiment. Fig 5 shows that biodegradation of heavy oil fractions caused lighter fraction to accumulate, hence reducing oil viscosity and interfacial tension (IFT). Viscosity reduction in a range of 11 – 22% ($P \approx 0.05$) occurred in all treatment and high viscosity reduction was observed in DS1 and DS3 treatment. Meanwhile, IFT reduction of 21 – 32% ($P \approx 0.05$) occurred and high IFT reduction was observed in DS31 and DS1 treatment.

Phylogenetic Analysis and Identification using 16S rRNA Sequences. Identification of isolates using 16S rRNA approach showed that isolate DS1, DS3, and DS31 were most closely related to *Bacillus* sp strain

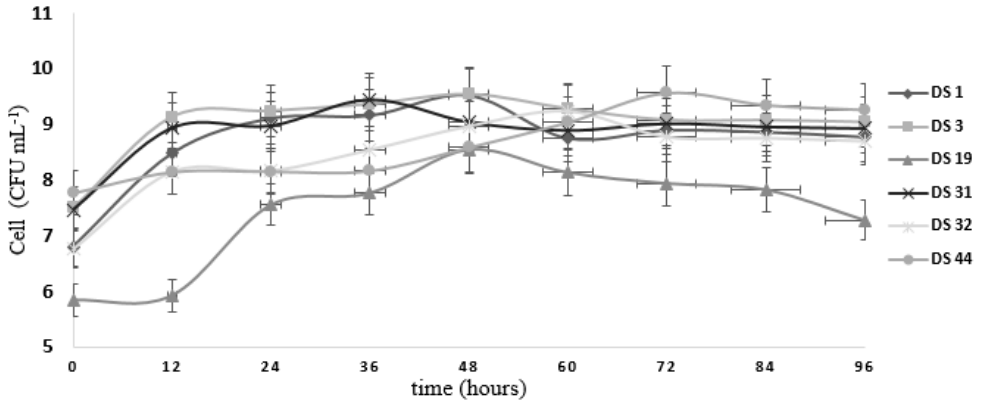

Fig 1 Growth of bacterials coded DS1, DS3, DS19, DS31, DS32, and DS44 at different periods (h) incubation ($P \approx 0.05$).

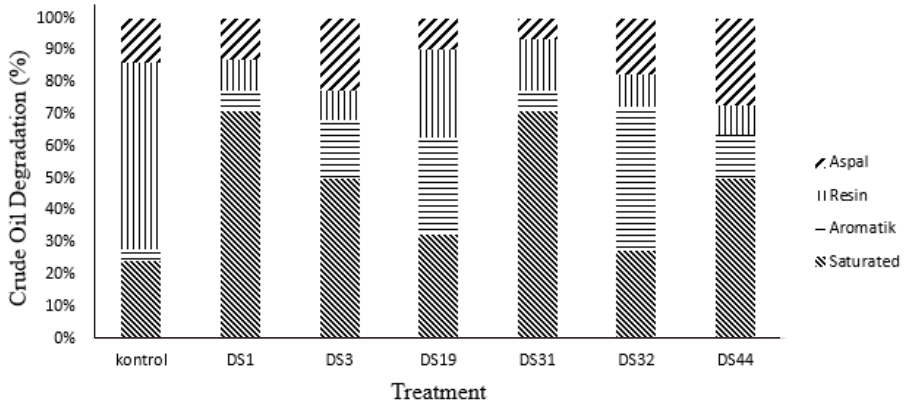

Fig 2 Abundance percentage of SARA Fraction after 7 days treatment with selected bacterial isolates coded DS1, DS3, DS19, DS31, DS32, and DS44.

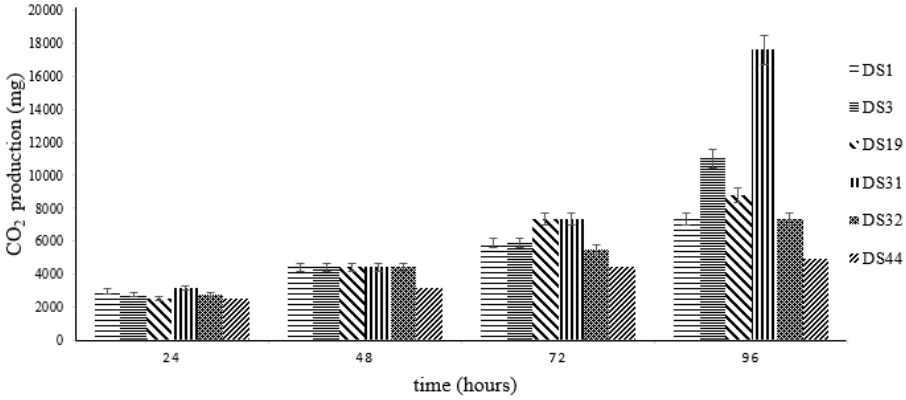

Fig 3 Carbon dioxide released (mg) during biodegradation of crude oil in a biometric flask by isolates coded DS1, DS3, DS19, DS31, DS32, and DS44 at different periods (h) incubation ($P \approx 0.05$).

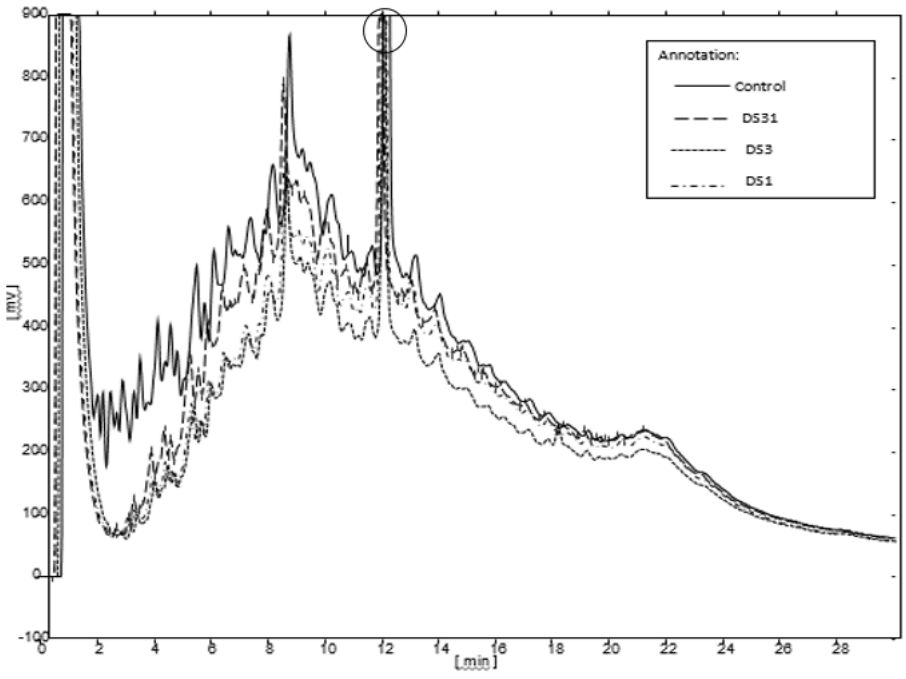

Fig 4 GC-MS analysis of biodegradation of untreated crude oil and treated crude oil by DS1, DS3 and DS31 (after 7 days of incubation). Based on GC-MS analysis, gained TPH content of control approximately 16,072 (μg g^{-1}) by calculation of residual saturated and aromatic fractions (showed by peak chromatogram). TPH content of treatment by DS1, DS3 and DS31 sequentially are 7,510 (μg g^{-1}), 8,040 (μg g^{-1}), and 8,020 (μg g^{-1}). The highest peak chromatogram is defined as internal standard on retention time 12 min (showed by black circle).

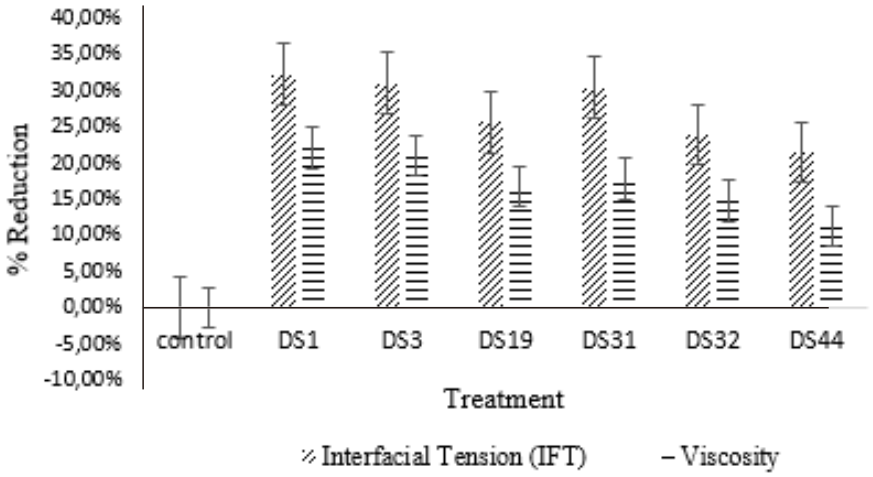

Fig 5 Interfacial Tension and Viscosity Reduction (%) after Seven Days Incubation Experiment ($P \approx 0.05$).

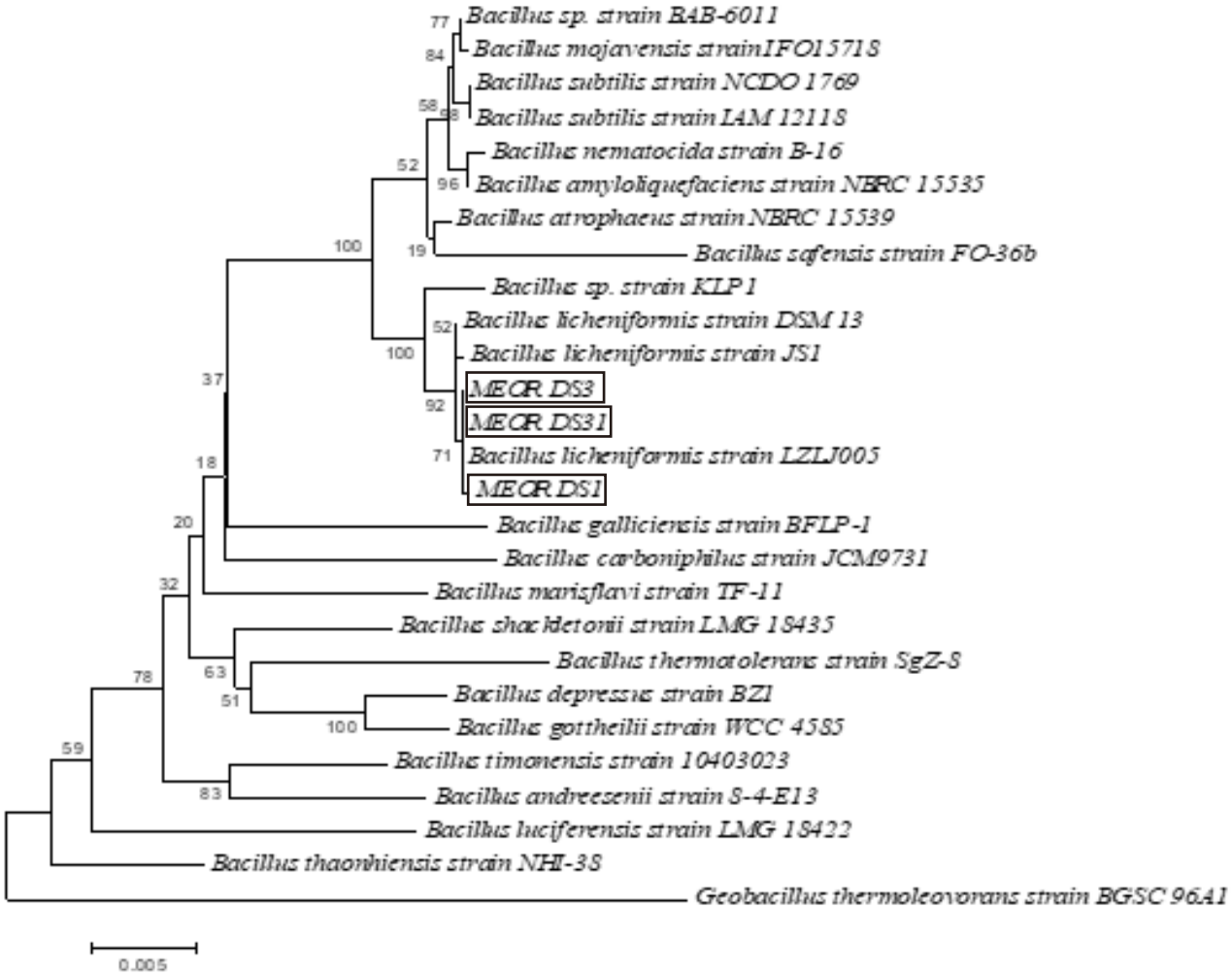

Fig 6 Phylogenetic tree of isolates DS1, DS3 and DS31 based on 16S rRNA gene sequencing analysis constructed by Neighbor-Joining. Isolates coded DS1, DS3 and DS31 were identified using BLAST. Based on BLAST result from database NCBI (http://www.ncbi.nlm.nih.gov) showed that isolate DS1 has identity 99% and query cover 100% (NR118996.1), isolate DS3 has identity 99% and query cover 99% (KR018738.1), and isolate DS31 has identity 99% and query cover 99% (JX068644.1).

KLP1 (Fig 6). Based on phylogenetic tree constructed, it could be seen that isolate DS1, DS3, and DS31 were present in the same clade as *Bacillus licheniformis* (Fig 6).

DISCUSSION

Petroleum is a complex mixture of hydrocarbon and is generally classified into four groups: saturates, aromatics, resins, and asphaltenes. The convenience of microbes in degrading four classes of hydrocarbon fractions also varies. The vulnerability of microbial to degrade hydrocarbons can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes. Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all (Das and Chandran 2011). Therefore, biodegradation of crude oil could only be completed by different microbes with special functions. Sequential isolation was performed to obtain hydrocarbonoclastic bacteria with various ability to degrade different fractions of oil. The results of sequential isolation from of South Sumatera's well were grouped according to the stages of isolation. Stage I was expected to isolate light oil fraction-degrading bacteria while stage II was expected to isolate heavy oil fraction-degrading bacteria. Stage I and II obtained 12 and 19 isolates respectively.

Resazurin has a non-flouresen blue color that can be reduced to pink. The use of this dye is based on its ability as an intermediate electron acceptor in the electron transport chain without interrupting normal electron transport. Resazurin assay indicated oil degrading ability because this assay could screen oxygenase enzyme activity of hydrocarbonoclastic bacteria. When O_2 in growth media decreases as it is used by enzyme oxygenase in degradation hydrocarbon fraction, resazurin will be reduced and change color from purple to nones. This indicates high oxygenase enzyme activity in biodegradation process of hydrocarbon fraction. Screening test by resazurin assay indicated that DS1, DS3, DS19, DS31, DS32 and DS44 have high oil degrading ability because they could use oxygen as inisiator for hydrocarbon oxidation by oxygenases complexes enzyme. The initial intracellular attack of hydrocarbon is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases enzyme. Peripheral degradation pathways convert hydrocarbon step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle (Benedek *et al*. 2010).

Crude-oil degrading ability was significantly affected by growth of oil-degrading bacteria. Fig 1 and Fig 2 showed that there was a correlation between optimum growth-rate and degradation of SARA fraction, especially for degradation of heavier crude oil fraction. Mostly, all selected isolates, exhibited the same pattern of crude oil degradation. Firstly, the isolates degraded the most readily degradable hydrocarbons. As their concentration decreased and only heavy oil fraction remained, the degradation rate slowed down. Therefore, the composition ratio of hydrocarbons changes when degradation rate of some oil hydrocarbons exceeds the degradation of others (Gailiūtė *et al.* 2014).

This research exhibited degradation of crude oil altered SARA composition of crude oil. SARA analysis of crude oil was used to examine the changes of oil fractions before and after degradation. This method can determine the relative content of each fraction in the oil sample (Guizhou *et al.* 2013). Degradation of hydrocarbon fractions can be occurred because of other mechanisms involved are (1) attachment of microbial cells to the substrates and (2) production of biosurfactants, besides mediated by spesific enzyme (Das and Chandran 2011). Biosurfactants that produce by microbials can act as emulsifying agents by decreasing the surface tension and forming micelles. The microdroplets encapsulated in the hydrophobic microbial cell surface are taken inside and degraded (Bordoloi and Konwar 2009). As seen on Fig 5 there is an interfacial tension (IFT) reduction of crude oil after incubation experiment treated by six tested isolates. Reduction of IFT can occurred through production of biosurfactant by each isolates to mediated degradation of insoluble hydrocarbon fraction. Isolate DS31 that was obtained from isolation II showed highest ability to degrade resins and asphaltenes fraction, leaving only few resins fraction and less asphaltenes fraction unutilized (Fig 2). In addition, isolate DS1 and DS3 showed high ability to degrade aromatic fraction and were able to increase the accumulation of saturated fraction after degradation of heavy hydrocarbon fractions. Accumulation of light crude oil fraction reduce oil viscosity (Fig 5), which increase its mobility to production well. From this research, known that degradation of crude oil can affect chemical and physical characterization of crude oil (i.e. increment of light crude oil fraction and declining of IFT and

viscosity crude oil).

Chemical change of crude oil was observed through mineralization test using biometric system and GC-MS analysis. Fig 3, showed that isolate DS31 produced the highest concentration of CO_2 (17600 mg). CO_2 production by hydrocarbonoclastic microorganisms is produced through mineralization of hydrocarbon by microbial extracellular enzyme i.e. mono- and dioxygenases (Maier 2008). Fig 4, showed that new saturated and aromatics compounds. These new compounds are calculated and gained lower total petroleum hydrocarbon than untreated crude oil. Based on chemical and physical characterization test, isolate DS1, DS3 and DS31 were concluded to have the highest oil degrading abilities.

Phylogenetic analysis, showed the majority of the selected bacteria belonged to different strain *Bacillus licheniformis* (Fig 6). *Bacillus licheniformis* is a Gram positive bacteria, and has the ability to produce lipopeptide biosurfactant (García-Alcántara *et al.* 2016). Previous studies revealed that various species of *Pseudomonas* and *Bacillus* are common inhabitants of petroleum ecosystems (Munawar *et al.* 2012; Ismail *et al.* 2013; Guizhou *et al.* 2013; Gailiūtė *et al.* 2014).

However, studies *B. licheniformis's* ability to degrade crude oil, especially from South Sumatera were limited and no reports exist for these strains. Bioinformatics analysis using KEGG (http://www.genome.jp/) *B. licheniformis* could degrade benzene, xylene, styrene, dioxin, chlorobenzene, chlorocyclohexane, naphthalene and benzoate compounds. This ability was affected by its ability to produce of biosurfactant. Biosurfactants have the ability to reduce surface tension through solubilisation of fatty acids present in crude oil which leads to proficient exploitation of hydrocarbon by microbes. Therefore, biosurfactant production allowed the utilization of hydrocarbons by microorganisms, reduction of IFT, and implementation of other MEOR potential in oil industry (Bordoloi and Konwar 2009; Lazar *et al*. 2007).

This great potential has also been investigated by García-Alcántara *et al. (*2016) who reported that *B. licheniformis* consortium was 6 times higher compared with that obtained with five oil-degrading microorganisms: *Achromobacter (Alcaligenes) xylosoxidans*, *B. cereus*, *B. subtilis, Brevibacterium luteum,* and *Pseudomonas pseudoalcaligene*s.

This research, demonstrated that sequential isolation method could successfully obtain hydrocarbonoclastic bacteria that can could degrade saturated, aromatic, resin, and asphaltene fractions. In addition, isolates with the highest ability to degrade crude oil DS1, DS3 and DS31, were identified as different strains of the species *Bacillus licheniformis*. High crude-oil degrading ability was confirmed by SARA analysis, CO_2 production, IFT and viscosity measurement and GC-MS analysis. Degradation of heavy crude oil (resin fraction) altered physical characteristic of crude oil as indicated by reduction IFT and viscosity 17-31% and chemical characteristic of crude oil as seen by an increment of lighter crude oil fractions, and high CO_2 production. This study suggested that isolate DS1, DS3 and DS31 has a high potential to be used in MEOR.

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REFERENCES

Biodia ED, Montagnolli RN, Lopes PRM. 2010. Microbial biodegradation potential of hydrocarbons evaluated by colorimetric technique: a case study. Appl Microbiol Biotechnol. 7: 1277-1288.

Benedek T, Máthé I, Táncsics A, Lányi S, Márialigeti K. 2010. Investigation of hydrocarbon-degrading microbial communities of petroleum hydrocarbon contaminated soils in Harghita county, Romania. Romania. Scientific Bulletin: Mining, Mineral Processing, Non-ferrous Metallurgy, Geology and Environmental Engineering-North University of Baia Mare. 24(2):15-24. doi:10.4172/2157-7463.1000163.

Brown LR. 2010. Microbial Enhanced Oil recovery (MEOR). Curr Opi Microbiol. 13(3): 316-320. doi: 10.1016/j.mib.2010.01.011.

Brown AE. 2001. Microbiological applications laboratory manual in general microbiology. McGraw Hill Companies.

Bordoloi NK, Konwar BK. 2009. Bacterial biosurfactant in enhancing solubility and metabolism of petroleum hydrocarbons. J Hazard Mater. 170(1): 495-505. doi: 10.1016/j.jhazmat.2009.04.136.

Cappucino J, Sherman N. 2008. Microbiology: A laboratory manual. Benjamin-Cummings Pub Co.

Das N, Chandran P. 2011. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol Res Int. 9(13):1-13. doi: 10.4061/2011/941810 .

Gailiūtė I, Račkauskienė G, Grigiškis S. 2014. Changes in crude oil hydrocarbon composition during

biodegradation by *Arthrobacter* sp. M1 and *Acinetobacter* sp. Pr82 in selected optimal conditions. Biologija. 60(3). doi: 10.6001/biologija.v60i3.2972.

García-Alcántara JA, Maqueda-Gálvez AP, Téllez-Jurado A, Hernández-Martínez R, Lizardi-Jiménez MA. 2016. Maya Crude-Oil Degradation by a Bacillus licheniformis Consortium Isolated from a Mexican Thermal Source Using a Bubble Column Bioreactor. Water Air Soil Pollut. 227(11): 413. doi: 10.1007/s11270-016-3121-7.

Gudiña EJ, Pereira JF, Rodrigues LR, Coutinho JA, Teixeira JA. 2012. Isolation and study of microorganisms from oil samples for application in microbial enhanced oil recovery. Int Biodeterior Biodegrad. 68:56–64. doi: 10.1016/j.ibiod.2012.01.001.

Guizhou G, Zheng L, Dongfeng Z, Chaocheng Z. 2013. Isolation and characterization of a thermophilic oil-degrading bacterial consortium. China Petroleum Proc Petrochem Technol. 15(2): 82-90.

Hao R, Lu A, Wang, G. 2004. Crude-oil Degrading Thermophilic Bacteria Isolated from An Oil Field. J Microbiol. 50(3): 175-182. doi: 10.1139/w03-116.

Halim AY, Andromeda A, Mustafa I, Haro M, Purwasena IA, Juli N, Siregar S. 2008.The Laboratory Study of Indigenous Bacterial Activities on Physical Oil Sample Properties from West Java Oil Reservoir: Implication for MEOR (Microbial Enhanced Oil Recovery) Application. Simposium Nasional dan Kongres X. Ikatan Ahli Teknik Perminyakan Indonesia.

Ismail W, Alhamad NA, El-Sayed WS, El Nayal AM, Chiang YR, Hamzah RY. 2013. Bacterial degradation of the saturate fraction of Arabian light crude oil: biosurfactant production and the effect of ZnO nanoparticles. J Pet Environ Biotechnol, 4(6):163. doi:10.4172/2157-7463.1000163.

Janda JM, Abbot SL. 2007. 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils and Pitfalls. J Clin Microbiol. 11(7): 2761-2764. doi:10.1128/JCM.01228-07.

Lazar I, Petrisor IG, Yen TF. 2007. Microbial enhanced oil recovery (MEOR). Petrol Sci Technol. 25(11): 1353-1366. doi: 10.1080/10916460701287714.

Long YF, Wang WH, Xu YL, Gao T, Wu QY, Zhu ZY, Wei XJ. 2013. The research on bio-chemical combination drive and its application in Y9 reservoir in block ZJ2 of Jing'an oilfield. J Oil Gas Technol. 35(9):142–5.

Madigan MT, Martinko JM, Stahl DA, Clark, DP. 2014. Brock Biology of microorganisms. Benjamin Cummings.

Maier RM, Pepper IL, Gerba CP, Gentry, T. 2008. Environmental Microbiology. Elsevier.

Maudgalya S, Knapp RM, Mclnerney, MJ. 2007. Microbial enhanced-oil-recovery technologies: a review of the past, present, and future. SPE. 4(3): 2-11. doi: 10.2118/106978-MS.

Muggeridge A, Cockin A, Webb K, Frampton H, Collins I, Moulds T, Salino P. 2014 Recovery rates, enhanced oil recovery and technological limits. Phil Trans R Soc. A 372: 20120320. doi:10.1098/rsta.2012.0320.

Muhammad I, Tijjani N, Dioha IJ, Musa A, Sale H, Lawal AM. 2013. SARA Separation and Determination of Concentration Levels of Some Heavy Metals in Organic Fractions of Nigerian Crude Oil. Chem Mater Res. 3(4): 7-14. doi: 10.1080/10916466.2016.1163397.

Munawar, Aditiawati P, Astuti, DI. 2012. Sequential isolation of saturated, aromatic, resinic and asphaltic fractions degrading bacteria from oil contaminated soil in South Sumatera. Makara J Sci. 58-64. doi: 10.7454/mss.v16i1.1282.

Patel J, Borgohain S, Kumar M, Rangarajan V, Somasundaran P, Sen R. 2015. Recent developments in microbial enhanced oil recovery. Renew Sustain Energy Rev. 52: 1539-1558. doi: 10.1016/j.rser.2015.07.135.

Ruxia H, Anhuai LU. 2007. Conversion and degradation of crude oil by *Bacillus* SP3. Chin J Geochem. 26(2): 201-206. doi: 10.1007/s11631-007-0201-9.

Safdel M, Anbaz MA, Daryasafar A, Jamialahmadi M. 2017. Microbial enhanced oil recovery, a critical review on worldwide implemented field trials in different countries. Renew Sustain Energy Rev. 74:159-172. doi: 10.1016/j.rser.2017.02.045.

Santos RG, Loh W, Bannwart AC, Trevisan OV. 2014. An overview of heavy oil properties and its recovery and transportation methods. Braz J Chem Eng. 31(3): 571-590. doi: 10.1590/0104-6632.20140313s00001853.

Sen R. 2008. Biotechnology in petroleum recovery: the microbial EOR. Prog Energy Combust Sci. 34(6):714–24. doi: 10.1016/j.pecs.2008.05.001.

Seo JS, Keum YS, Li QX. 2009. Bacterial Degradation of Aromatic Compunds. Int J Environ Res Public Health. 6(1):278-309. doi: 10.3390/ijerph6010278.

Shah A, Fishwick R, Wood J, Leeke G, Rigby S, Greaves M. 2010. A review of novel techniques for heavy oil and bitumen extraction and upgrading. Energy Environ. Sci. 3(1): 700-714. doi: 10.1039/B918960B.

Sharpley JM. 1966. Elementary petroleum microbiology. Gulf Publishing Company.

Sheng JJ. 2013. Enhanced oil recovery field case studies. Elsevier.

Shoeb E, Ahmed N, Akhter J. 2015. Screening and characterization of biosurfactant-producing bacteria isolated from the Arabian Sea coast of Karachi. Turk J Biol. 39(1):210-216. doi: 10.3906/biy-1405-63.

Speight JG. 1991. The chemistry and technology of petroleum. Marcel Dekker, Inc.

Tamura K, Nei M, Kumar S. 2004. Prospects for inferring

very large phylogenies by using the neighbor-joining method. Proc Nat Acad Sci USA. 101(30):11030-11035. doi: 10.1073/pnas.0404206101.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 28(10):2731-2739. doi:10.1093/molbev/msr121.

Yan P, Lu M, Yang Q, Zhang HL, Zhang ZZ, Chen R. 2012. Oil recovery from refinery oily sludge using a rhamnolipid biosurfactant-producing Pseudomonas. Bioresour Technol. 116:24–8. doi: 10.1016/j.biortech.2012.04.024.

Youssef N, Simpson DR, McInerney MJ, Duncan KE. 2013. In-situ lipopeptide biosurfactant production by Bacillus strains correlates with improved oil recovery in two oil wells approaching their economic limit of production. Int Biodeterior Biodegrad. 81(1):127-132. doi: 10.1016/j.ibiod.2012.05.01.