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# Isolation of deep-sea sediment bacteria for oil spill biodegradation

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**Abstract**. The potency of deep-sea sediments bacteria is still unfamiliarly used for oil spill biodegradation, and this research was to isolate and identify bacteria from those sediments and adapt them in oily media. Bacterial isolates were cultivated and adapted in a mixture of 0.1% v/v crude oil media. Seven deep-sea sediment samples were treated to isolate bacteria and it produced a variety of bacteria population 5.5 x $10^2$ CFU $mL^{-1}$ to 1.5 x $10^6$ CFU $mL^{-1}$. These populations apparently increased after cultivation and adaptation and gave a varying population from 3.0 x $10^6$ CFU $mL^{-1}$ to 6.8 x $10^8$ CFU $mL^{-1}$. The increased bacteria population was an indication of the bacterial capability of using carbon in crude oil as a substrate. Those bacteria were *Raoultella* sp., *Enterobacter* sp. and *Pseudomonas* sp.
**Key words**: Bacteria, biodegradation, deep-sea sediment, isolation, oil spill.

**Abstrak**. Potensi bakteri sedimen laut dalam untuk teknologi biodegradasi tumpahan minyak masih belum banyak dimanfaatkan. Tujuan dari penelitian ini adalah mengisolasi dan mengidentifikasi bakteri alami dari contoh sedimen laut dalam, dan menyesuaikannya dalam media yang berminyak. Isolat bakteri dikultivasi dan diadaptasi menggunakan media air laut terkondisi nutrien yang dicampur dengan minyak mentah 0.1% v/v. Isolasi dari 7 contoh sedimen laut dalam menghasilkan jumlah populasi bakteri 5.5 x $10^2$ CFU $mL^{-1}$hingga 1.5 x $10^6$ CFU $mL^{-1}$. Setelah kultivasi dan adaptasi dilakukan, populasi bakteri meningkat yaitu sebesar 3.0 x $10^6$ CFU $mL^{-1}$ hingga 6.8 x $10^8$ CFU $mL^{-1}$. Peningkatan tersebut mengindikasikan bahwa bakteri mampu menggunakan karbon pada minyak mentah sebagai sumber nutrisi. Bakteri yang teridentifikasi dalam penelitian ini diantaranya *Raoultella* sp., *Enterobacter* sp. dan *Pseudomonas* sp.
**Kata kunci**: Bakteri, biodegradasi, isolasi, sedimen laut dalam, tumpahan minyak.

**Rezumat**. Capacitatea bacteriilor din sedimentele din profunzimile marine este încă puţin utilizată pentru biodegradarea deversărilor de petrol, iar acest studiu vizează izolarea şi identificarea bacteriilor din aceste sedimente, şi adaptarea lor la mediul petrolier. Probe bacteriene au fost cultivate şi adaptate într-un amestec de 0.1% v/v mediu petrolier brut. Şapte probe sedimentare din profunzimile marine au fost folosite pentru a izola bacteriile şi au rezultat o varietate de populaţii bacteriene de la 5.5 x $10^2$ CFU $mL^{-1}$ la 1.5 x $10^6$ CFU $mL^{-1}$. Aceste populaţii s-au înmulţit evident după cultivare şi adaptare, şi au ajuns la o variaţie de la 3.0 x $10^6$ CFU $mL^{-1}$ la 6.8 x $10^8$ CFU $mL^{-1}$. Amplificarea populaţiei bacteriene a indicat capacitatea bacteriană în ce priveşte folosirea carbonului din petrolul brut ca şi substrat. Aceste bacterii au fost specii de tipul *Raoultella* sp., *Enterobacter* sp. şi *Pseudomonas* sp.
**Cuvinte cheie**: bacterii, biodegradare, sediment din profunzimi marine, izolare, deversare petrolieră.

**Introduction**. Oil spills at sea derived from tanker accidents, broken oil pipe, oil drilling have generated severe pollution of the sea. In general, the oil spill in the sea encounters a variety of natural processes such as evaporation, emulsification, dispersion, photooxidation, biodegradation, and sedimentation (US EPA 2014). However, the accumulations of contaminants introduced into the marine environment are much faster compared to the rate of their recovery processes. Consequently, an application of technology such as bioremediation is required to address oil pollution in marine environment (Nugroho 2006).

Bioremediation as a technique of using petroleum degrading bacteria is a relatively inexpensive, efficient solution and easy to apply, besides no further impact to marine environment (Thapa et al 2012). The utilization of local marine bacteria from the water column has been tested in various environments (Nashikin & Shovitri 2013; Darmayati 2009; Nababan 2008). Naturally, bacteria are able to degrade complex hydrocarbons such as long chain hydrocarbons (*n*-alkane) and polyaromatic hydrocarbon (Sari 2007). The bacteria use hydrocarbons as a source of carbon and energy to develop (Thapa et al 2012). Some examples of degrading bacteria are *Pseudomonas aeruginosa, Bacillus subtilis,* and *Acinetobacter lwoffi*, which were able to degrade aliphatic crude oil by 77.8%, 76.7%, and 74.3%, respectively (Al-Wasify & Hamed 2014).

Sediment as a source of bacterial isolates is a complex habitat with a number of organic materials lucrative for growing bacteria. The organic material in marine sediments derives from the residual feed, decomposed dead animals and plants, and nutrients particles precipitated by their gravity (Rampen et al 2012). Hence, the sediment has more potential content of bacteria compared with sea water. This study was to isolate and identify bacteria from deep-sea sediments and to test their adaptation in oily environment.

## Material and Methods

***Sediment sampling***. Deep sea sediment samples from seven locations at ~ 1000 meter depth of Indonesian Makassar Strait to Flores Sea were collected using gravity cores in July-August 2011 (Figure 1). All core samples were then kept in at 4 ºC during transportation and their storage in the laboratory for further analysis.

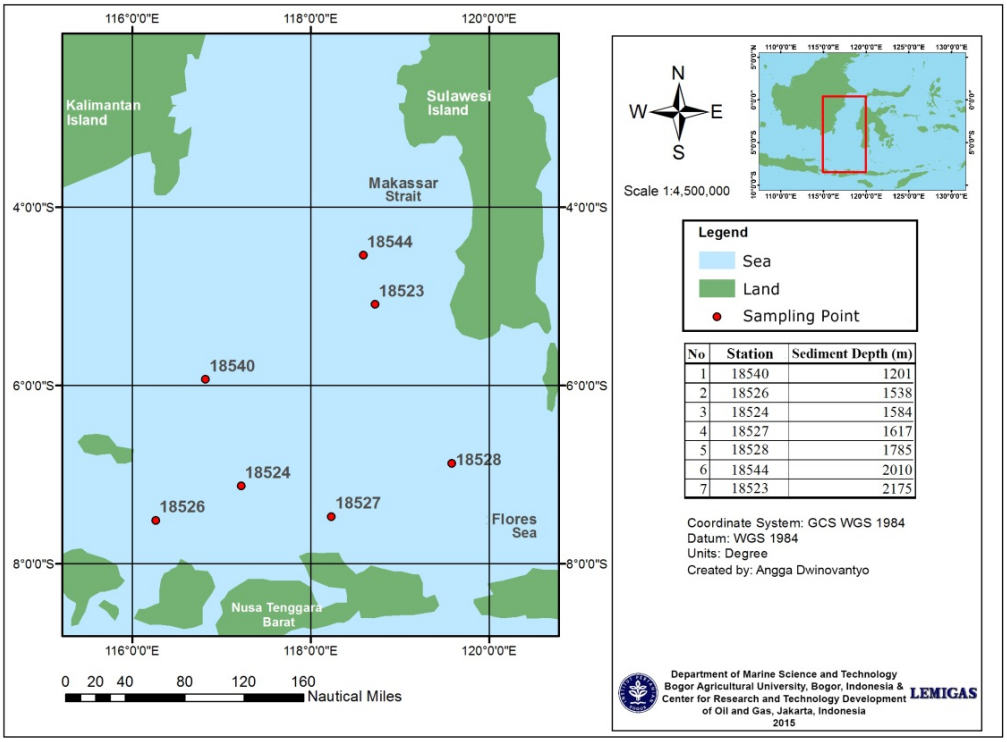


Figure 1. Location Map of the deep-sea sediment sampling.

***Preparation of inoculum***. The method is based on the procedure developed by Ahmed et al (2014). 1 g of freeze-dried sediment sample was put into a sterile test tube and then homogenized using a vortex shaker. The mixture was left to allow the sediment precipitate from the water, in which the bacteria inoculum was extracted for further bacteria isolation.

***Isolation of crude oil degrading bacteria***. The isolation was based on the method developed by Mendham et al 2000 and Atlas 2010. 1 mL of the inoculum containing water was pipetted and transferred into a test containing 9 mL physiological solution (NaCl 9%) and mixed. The mixture was consecutively diluted three times with ratios of 1:9 for each dilution tubes. 0.1 ml of each mixture tube was pipetted and transferred into four Petri dishes containing 10-15 mL plate count agar (PCA). These four Petri dishes were incubated for three days to allow bacteria to develop. All of these processes were conducted in a laminar flow bench to avoid contamination. Total plate count (TPC) was determined by colony-forming-unit $mL^{-1}$ (CFU $mL^{-1}$) (APHA 2012).

***Bacterial cultivation***. Bacteria were developed by collecting isolate from Petri dish and transferred in 100 mL nutrient broth (NB) liquid medium. The culture was shaken at 120 rpm for 48 hours at room temperature. The bacteria growths were signed by changes of color and being more turbid in the liquid media. 0.1 mL of this culture was poured into the Petri dish and left for three days incubation (Okoro 2010). The bacterial populations were counted by TPC (APHA 2012).

***Bacterial adaptation***. 10 mL bacterial culture aliquot and crude oil (concentration 0.1% v/v) were poured into 100 mL nutrient-conditioning sea water. The composition of nutrient was 1.26 g $MgSO_4 \cdot 7H_2O$, 1 g KCl, 2.5 g $KH_2PO_4$, 3.75 g $Na_2HPO_4$, and 1.29 g $NaNO_3$ dissolved in 3 liter sterile sea water (Okoro 2010). The mixture was shaken at 120 rpm for 72 hours in room temperature. Bacteria population was counted by optical density (Razika et al 2010). When the population was $>10^6$ CFU $mL^{-1}$, the bacteria could be used for biodegradation processes (Okoro 2010).

***Bacterial identification***. Identification of isolated bacteria was based on the Biolog Gen III identification system (Biolog Inc., USA). Pure isolates of the adapted bacteria were developed into a nutrient agar (NA) medium. This media was then transferred into a solution of G-negative bacteria inoculation fluid. This solution was measured by Biolog turbidimeter to indicate bacterial content. 150 µL of this solution was then pipetted and put into the GN2 MicroPlate™ and incubated for 16-24 hours. The plate was determined by MicroStation Microplate Reader. The identification process was run by a pre-loaded database ID on a computer. Bacterial species appeared on the monitor in the form of 1-10 species that have an adjacent reaction pattern with the percentage of similarity (Wragg et al 2014).

**Results and Discussion**. The visual characteristics of the marine sediments were green, dark green, brownish-green, and dark brown (Table 1). This could reflect the extent of sedimentary oxidation; brown color was more oxidative; although no relationship between the depth of sediment and the oxidation level was observed.

Table 1

Visual characteristics of marine sediment in each station

| No | Station | Depth (m) | Characteristics of sediment |
|---|---|---|---|
| 1 | 18540 | 1201 | Green mud |
| 2 | 18524 | 1584 | Dark green mud |
| 3 | 18544 | 2010 | Brownish-green mud |
| 4 | 18528 | 1785 | Dark green mud |
| 5 | 18527 | 1617 | Brownish-green mud |
| 6 | 18526 | 1538 | Green mud |
| 7 | 18523 | 2175 | Dark brown mud |

The number of bacteria isolated varied from the highest bacterial population of 1.5 x $10^6$ CFU $mL^{-1}$, which was obtained at the Station 18540 to the lowest, 5.5 x $10^2$ CFU $mL^{-1}$, which was obtained at the Station 18523 (Figure 2). The differences in number of population at each station could possibly be related to the availability of bacteria in sediment samples and sample handling.

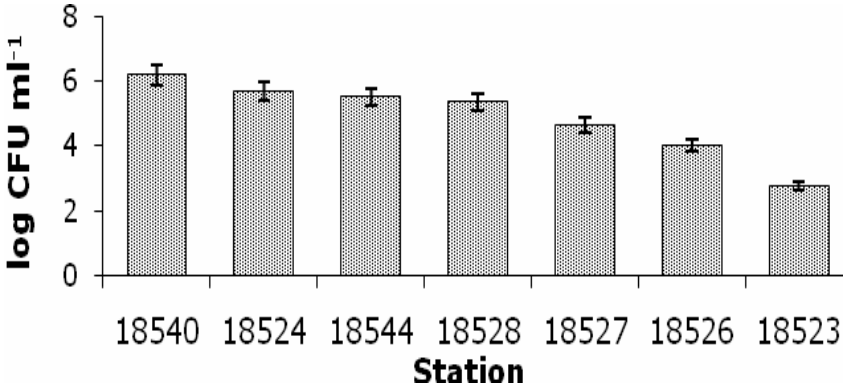


Figure 2. Total population of bacteria in 7 sediment samples.

Bacterial isolates were cultivated and adapted to environmental conditions containing small volume of oil. Cultivation processes were performed to regenerate and multiply the number of bacterial populations. The experiment showed that bacteria was able to develop after 48 hours, and those, visually, were indicated by the changes of color from pale yellow (as initial) to green or orange (Figure 3).

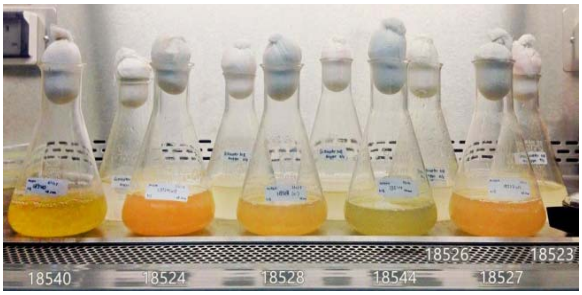


Figure 3. Color change in bacteria cultivation.

The color changes to yellow occurred in the samples of Station 18540, 18528, 18524, 18523; changing to orange occurred in the samples of Station 18524, 18527; and to green occurred in the sample of Station 18544. Color change indicated bacterial response to media as a result of secondary metabolites produced by bacterial pigments (Ahmad et al 2012). The increase of bacterial population would give more turbid and concentrated color in the solution, as shown in the sample solution of Stations 18540.

Bacterial adaptation in oily media (0.1% of crude oil) performed on all the cultivated bacteria was possibly used for the process of biodegradation. Bacteria were able to adapt to the media characterized by increasing the bacterial population from all sediment samples (Figure 4). The adaptation ability was likely related to the extent of the capability of their metabolism to use hydrocarbons (Okoro 2010). Hydrocarbons were used as a source of organic carbon in the metabolism (Nashikin & Shovitri 2013). It suggested that bacteria of marine sediment are likely suitable for biodegradation of crude oil.

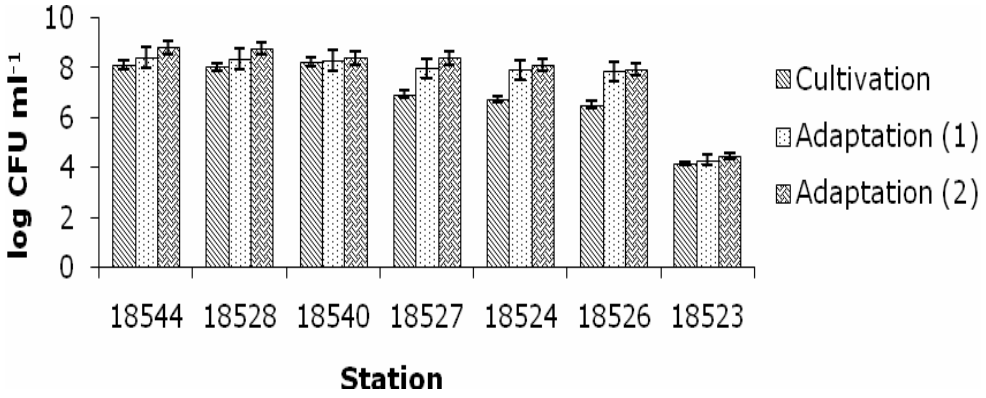


Figure 4. Bacterial population growth after cultivation and adaptation process.

Identification was only performed on bacterial isolates having the highest number of population (*e.g.* sample from Stations 18544). Bacteria identified during the study were *Raoultella* sp., *Enterobacter* sp. and *Pseudomonas* sp., which were attained through matrix-assisted laser desorption ionization-time of flight mass spectrometry used in Biolog GEN III identification system. Rodrigues et al (2008) reported that *Raoultella* could degrade toluene, xylene, naphthalene, and *n*-alkanes compounds. *Enterobacter* is able to degrade oil and used as a biosurfactant. *Enterobacter* effectively degraded crude oil at pH 7 and temperature of 30 ºC (Ahmed et al 2014). Dawson & Chang (1992) reported that *Pseudomonas* has a specific enzyme to break aliphatic and aromatic hydrocarbon compounds. These bacteria have been found in the Makassar Strait (Damayati 2009).

**Conclusions**. *Raoultella* sp., *Enterobacter* sp. and *Pseudomonas* sp. were predominantly found in seven deep-sea sediment samples with varying populations. These bacteria were able to adapt to the oily culture media and suggested to have potential for oil-biodegradation processes.

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