IDENTIFICATION OF POTENTIAL DIESEL OIL-DEGRADING BACTERIA ISOLATED FROM MANADO SEA PORT BASED ON 16S rRNA GENE

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ABSTRACT

Petroleum contamination and its derivate in ecosystem are considered as environmental threat all over the world. Some microorganisms exhibit potential to degrade hydrocarbon in contaminated environments. This study aims at identifying potential diesel oil-degrading bacteria grown on artificial media. Bacteria isolated from Manado Sea port were grown in nutrient agar containing artificial diesel oil plus salt water and diesel oil only, respectively. The growing bacteria were isolated and each of them was grown separately to obtain pure isolate. Three bacterial isolates namely AO2, OA3 and OA4 were identified using 16S rRNA gene as *Pseudomonas aeroginosa*, *Klebsiella oxytoca,* and *Citrobacter* sp, respectively.

Keywords: diesel oil, diesel oil-degrading bacteria, Manado Sea Port, 16S rRNA gene

IDENTIFIKASI BAKTERI YANG BERPOTENSI SEBAGAI PENDEGRADASI MINYAK DIESEL DI ISOLASI DARI PELABUHAN LAUT MANADO

ABSTRAK

Kontaminasi minyak bumi dan turunannya dalam ekosistem dianggap sebagai ancaman lingkungan di seluruh dunia. Beberapa mikroorganisme menunjukkan potensi yang dapat menurunkan hidrokarbon dalam lingkungan yang terkontaminasi. Penelitian ini bertujuan untuk mengidentifikasi bakteri yang berpotensi sebagai pendegradasi minyak yang tumbuh pada media buatan. Bakteri diisolasi dari pelabuhan laut Manado dan ditumbuhkan dalam media NA yang mengandung minyak diesel dengan penambahan air garam buatan dan minyak diesel tanpa air garam buatan. Bakteri yang tumbuh diisolasi dan masing-masing ditanam secara terpisah untuk mendapatkan isolat murni. Tiga isolat bakteri yaitu AO2, AO3 dan AO4 yang telah diidentifikasi menggunakan 16S rRNA gen secara berturut-turut adalah *Pseudomonas aeroginosa*, *Klebsiella oxytoca*, dan *Citrobacter* sp.

Kata kunci: minyak diesel, bakteri pendegradasi minyak diesel, Pelabuhan Laut Manado, gen 16S rRNA

INTRODUCTION

Petroleum refineries products are needed continously by modern society. However, accident spills of crude oil and its refined product occurs frequently during extraction, transportation, storage, refining, and distribution (Nikolopoulou *et al*., 2007). This can become serious threat to the environment. Disruption of ecosystem function by hydrocarbon spills among others are respiration and nitrogen cycle (Schafer *et al*., 2009). Human, as well as flora and fauna are endangered by the toxicity of the content of oil spill (van Gestel *et al*., 2001). According to Jusfah (1995), oil contamination does not only poison the living things in all ecosystems, it also disrupts light absorption for photosynthesis of aquatic plants.

Diesel oil is one of most widely used refined petroleum (Yuswono, 2008).

Continues low-level input of diesel oil accidentally from pipeline, tanker, or storage tank during refuelling creates an accute pollution problem. This spills are rarely noticed and will pose a serious threat to the environment due to accumulation of hydrocarbon contamination.

Marine shorelines serves a sa home to various number of organisms. Any hydrocarbon spills will have a great threats because of damaging capacity to the coastal environtment. Manado sea port is one of the busiest commercial sea port that connects islands of Sangihe, Talaud, and Maluku. This site has a high intensity of daily passangers embarkation due to its location in the central business of Manado city. During embarkation, refuelling, and docking time, diesel oil will be disposed aling with waste water into the sea which then in turn will affect surrounding marine ecosystem. This of course will lead to the pollution load of diesel oil spills around the port is increasingly rising.

There are several ways that can be done to overcome hydrocarbon pollution, namely physical, chemical and biological ways (Udiharto, 1992).The best biological way to mitigaze the polution load is to utilize existing oil degrading microorganisms in the environment. This way does not damage the environment and it is cost-effective. During exposure to hydrocarbon, dynamic composition and functional of marine microbial communities are altered. This favors bacteria that are able to utilize rich carbon source for they have hydrocarbon degradation capacity (Scott *et al*., 2014). Based on the reasons and facts described above, this study aims at identifying the potential diesel oil-degrading bacteria using partial 16S rRNA gene.

METHODS

This research was conducted from March 2014 to May 2014. Sampling wastaken place in the waters of the port of Manado. Culture and identification of bacteria were conducted in the laboratory of Biotechnology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Sam Ratulangi.

Samples of bacteria that were suspected to have the potentialto degrade diesel oil were taken from the water of the Port of Manado using a sterilizedbottles.

One milliliter of sea water taken from manado seaport was poured onto NA with diesel oil which did not contain salt and spread evenly. Another 1 mL of water sample was poured onto NA with diesel oil which contained 0.9% salt and spread evenly. The media were incubated for 2 x 24 hours at 37ºC. Each colony was picked and streak onto the same prepared media and incubated for another 2 x 24 hours at 37ºC. The streak plate method produces individual colony on agar. Each pure colony was referred as isolate. Each isolate was transferred to a MacConkey media to start a pure culture. Each isolate grown in MacConkey then was transferred to KIA media for isolate selection.

Extraction of DNA was conducted using protocol provided by the company (InnuPREP DNA Mikro Kit – Analytik Jena AG). Each of pure isolates from KIA media was taken using Oose needle then put into Eppendorf containing 1 mL of NB and grown for 6 hours at 37ºC to reach a an exponential phase. Each tube then was centrifuged at 6000 rpm for 2 minutes and the supernatant was discarded. 300 µl of lysis buffer and 25 µl protein kinase were added into each tube and vortexed for 5 seconds. Eppendorf tubes then were placed in termoblock at 70⁰C temperature for 15 minutes. To bind the DNA, 200 µl of TBS was added into each tube and vortexed. Spin filters were added to receiver tubes. Samples were added into each spin filter and centrifuged at 12.000 rpm for 2 minutes. For washing the DNA, 500 µl of HS was added into each tube and centrifuged at 12.000 rpm for 1 minute. 750 µl of MS was added then again centrifuged at 12.000 rpm for 1 minute. The washing procedure was repeated twice.

To remove the alcohol, filtrate was removed and spin filter was added to receiver tube and centrifuged at maximum speed for 2 minutes. To elute the DNA, spin filter was added to elution tube and added with 200 of µl elution buffer. The tubes were incubated at room temperature for 1 minute and centrifuged at 8000 rpm for 1 minute. The filtrated was kept in freezer until used for DNA amplification process.

DNA amplification was done using Tpersonal-combi Thermo-cycler (Whatman Biometra-Germany). Primers used for PCR

process were universal namely Bact-F1 forward primer (10 mM) 5'-AGAGTTTGATC (A/C) TGGCTCAG-3' and Uni-B1 reverse primer (10 mM) 5'-GGTTAC (G/C) TTTGTTACGACTT-3' with a total PCR volume of 40 uL with the following reaction mixture (Tabel 1): Ready to Load (RTL) PCR beads, 1 uLof each primer, miliq water and 2 uL of the bacterial DNA.

Table 1. PCR reaction mixture

	Volume (μL)	Master mix (μL)
Firepol	8	40
Miliq water	28	140
Primer Bact-F1	1	5
Primer Uni-B1	1	5
DNA template	2	10
Total Vol.	**40**	**200**

Ready-to-load (RTL) PCR tubes with all the ingredients were subjected to the PCR condition as follows: 95^0C lead heating and DNA predenaturation at 95^0C for 2 min, followed by 35 cycles of (1) denaturation at 95^0C for 30 seconds, (2) Primer attachment (annealing) at 50^0C for 30 seconds, and (3) elongation of DNA at 72^0C for 90 seconds. Additional polymerization (final extension) was performed at 72^0C for 1 minute.

Amplified DNA was separated by electrophoresis in 1% agarose gel. A total of 4 uL of loading dye added into DNA. Electophoresis was done in 0.5 x TBE on 50 volts voltage for 10 minutes and followed by 100 volts for 20 minutes. Afterwards the gel was stained with 1 mg/mL ethidium bromide for 20 minutes prior to vizualisation. DNA fragments was visualized under UV light from UV-transiluminator. Documentation was done using the Digital Compact BioDoc (Whatman Biometra-Germany).

Amplified DNA fragments were sent to 1Base Malaysia for sequencing procedure. DNA sequencing results were subjected to editing using Geneious to remove unnecessary and unreadable sequences. The edited DNA sequences then were analyzed using BLAST which is available online at NCBI to search similar sequence from available species of bacteria deposited in GenBank.

RESULTS AND DISCUSSIONS

Bacteria which growed on nutrient agar (NA) with 1-5 % diesel oil (with or without salt) were transferred into MacConkey agar with 3% diesel oil + 0.9% salt. MacConkey agar is based on the bile salt-neutral red-lactose agar developed by MacConkey, and it is recommended for detection and isolation of Gram-negative bacteria. During fermentation of lactose, local pH dropped around the colony causing a color change to neutral red and followed by bile precipitation. Bile salts mixture and crystal violet inhibited Gram-positive cocci, thus serves as selective agents that only allows Gram-negative bacteria to grow (Figure 1). The bacteria growed on this medium was picked individually and grown on KIA slant media.

Figure 1. Bacterial growth on MacConkey medium with 3% diesel oil + 0.9 % salt

Kligler iron agar (KIA) was used to differentiate microorganism on the basis of lactose and glucose fermentation (with or without gas production) and hydrogen sulfide production.
Isolate AO2 is a nonfermenter organism therefore it is not capable of using glucose or lactose. This organism only uses solely amino acids/proteins. This is indicated by red color of the slant, and the color of the butt remains unchanged (Figure 2A).
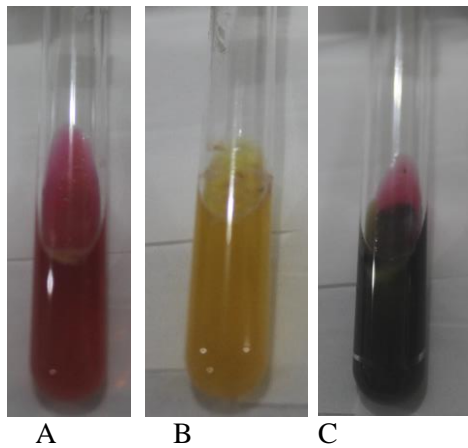
Figure 2. Reactions of microorganisms on KIA slant media containing. (A) Isolate AO2, (B) Isolate AO3, (C) Isolate AO4

Isolate AO3 is a glucose and lactose fermenter. Therefore caused the reaction to become acid over acid (A/A), which indicated that glucose and lactose have been metabolized.This bacterium quickly metabolized glucose producing an acid slant and an acid butt in a few hours (Figure 2B).

Isolate AO4 is a glucose fermenter, thus the tube reaction was alkaline over acid (K/A) signifying that only glucose is metabolized. This bacteria quickly metabolized glucose, initially produced an acid slant and acid butt (A/A) in few hours. After further incubation (18 hours) the glucose was consumed. Since the bacteria cannot use lactose, the amino acids were utilized as an energy source aerobically in the slant. Utilization of amino acids released ammonia (NH_3) thus increasing the pH, which turned yellow color into red. The butt remained acidic. It also produced H_2S (black color) (Figure 2C).

Figure 3 shows the amplified DNA visualized under UV light. The picture clearly shows that amplified DNA fragment of isolate AO2, OA3, and OA4 can be proceeded for sequencing due to its band quality.
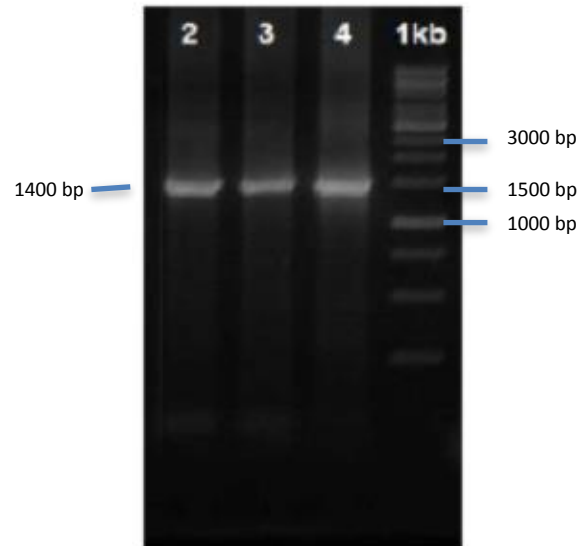

Figure 3. PCR analysis of 16S rDNA fragments on 1 % agarose gel DNA. Primers were able to amplify 1.4 kb fragment of 16S rDNA. Lane 2: isolate AO2; Lane 3: isolate AO3; Lane 4: isolate AO4. 1 kb: ExactMark 1 kb ladder.

Sequence of the three PCR products (AO2, AO3, AO4) obtained by applying primers BactF1 and UniB1 yielded only approximately 400 bases. This is due to poor quality of DNA amplification products. By performing a BLAST search within the GenBank database, the most similar sequences were identified. Isolate AO2 is highly similar (98%) with *Pseudomonas aeruginasa* strain TH-61 found in activated sludge of the biochemical pool of Dagang oilfield wastewater treatment plant in Beijing, China. Isolate AO3 is highly similar (98%) with *Klebsiella oxytoca* strain A2 isolated from oil-polluted soil in Tehran Iran. Isolate AO4 is highly similar (98%) with *Citrobacter* sp. isolated from oil-polluted soil in Tianjin China. According Rajasa (2004), if the percentage of homology ismore than 97% then it is the same species, between 93% and 97% belongs to the same genus with different species, and if less than 93% then there is the chance that it is a new species.

Mukherjee *et al*. (2010) reported that *Pseudomonas aeruginosa* isolated from crude oil at oil field in Assam, India, was capable to utilize hexadecane, benzene, or toluene as a sole carbon source aerobically. This bacterium produced extra cellular lipase that plays an essential role in hydrocarbon degradation, thus it is potential for

bioremediation of various hydrocarbon-contaminated environments.

Zang *et al*. (2005) assessed crude oil biodigredation potential of *Pseudomonas aeruginosa*. They found that this bacterium produced 15.4 g/L rhamnolipids when cultured in a basal mineral medium with glycerol as a sole carbon source. *Pseudomonas aeruginosa* is a gamma proteobacterium which is known to produce rhamnolipid and able to utilize and grow in high concentrations of crude oil. Rhamnolipid itself has been reported capable of stimulating biodegradation of hydrocarbons (Patel *et al*. 2014).Udochukwu *et al*. (2014) stated that *P. aeroginosa* is the predominant species in petroleum degradation and always found in every oil pollution site analyzed.

Chamkha *et al*. (2011) successfully isolated *Klebsiella oxytoca* strain BSC5 from an off shore oil field in Tunisia. Phylogenetic analysis of 16S rRNA gene sequence revealed that it has 99% sequence similiry with other *Klebsiella oxytoca*. This strain was capable of degrading a wide range of aliphatic hydrocarbons from C13 to C30. Another finding showed that *K. oxycota* isolated from crude petroleum oil samples in Saudi Arabia was able to utilize four different PAHs, namely phenanthrene, fluoranthene, pyrene, and benzene. This bacterium utilized almost 83% of benzene after 48 hrs of shaking if supplemented with nitrogen source (Mohamed *et al*., 2012).

According to Singh and Lin (2008), their isolate *Citrobacter freundii* (MRC$_3$) was able to degrade 86.94% diesel oil in two weeks. The bacterium was isolated from diesel-contaminated soil samples collected from a transport company in Kwazulu-Natal, South Africa.

According to Wu *et al*. (2013), to effeiciently degrade polycyclic aromatic hydrocarbons (PAH) in oil-contaminated soil a consortium of many different bacterial species is required. They obtained six PAH-degrading microbial consortia from oil-contaminated soils, and *Pseudomonas aeruginosa, Klebsiella oxycota*, and *Citrobacter* sp. are included. Erdoğan *et al*. (2011) found 33 strains of bacteria capable of degrading hydrocarbon. Those bacteria isolated from contaminated soil in Adana, Batman, and Adiyaman, Turkey among

others were *Pseudomonas aeruginosa, Klebsiella pneumonia, Citrobacter koseri*, and *Citrobacter amalonaticus.*

In order to achieve optimum degradation of hydrocarbon, an immobilized microbial consortium is suggested here, which comprises of a synergistic mixture of bacterial strains. Bacterial isolates found in this research can be used for finding a microbial consortium formulation, preferably using a biodegradable and economically cheaper immobilization support such as coconut fiber.

CONCLUSION

Bacterial isolates consortium that have the potential to degrade diesel oil has been isolated from Manado seaport. Three bacterial isolates namelyAO2, AO3 and AO4 were identified using partial 16S rRNA gene as *Pseudomonas aeroginosa*, *Klebsiella oxytoca,* and *Citrobacter* sp, respectively.

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