Molecular identification of bacteria and its potential for bioremediation of waste lubricant oil-polluted soil

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Abstract. The number of vehicles and machines increases every year around the world, positively correlated to the increasing demand of lubricant oil. However, hydrocarbon contained in the waste lubricant oil could contaminate the environment that crucially be recovered. Bioremediation is a promising solution since it is low cost and risk. Bacteria is a group of microorganism that mostly involved in bioremediation. Previously, we collected waste lubricant oil-contaminated soil from several service stations in West Sumatra. *In vitro* experiment indicated thirteen isolates have potential to degrade hydrocarbon containing in waste lubricant oil. In this study, the objectives are to identify the potential bacteria isolates molecularly as well as doing experimental study. We recognized 12 species of potential bacteria, i.e. *Alcaligenes* sp*, Comamonas testosterone, Bacillus thuringiensis, Bacillus amyloliqueficiens, Pseudomonas aeruginosa, Bacillus pseudomycoides, Brevibacterium* sp, *and Achromobacter* sp. These bacteria are cosmopolitan*. In situ* experiment using *Bacillus* sp, *Alcaligenes* sp and manure compost showed all types of treatment have significant different with control group to degrade total petroleum hydrocarbon (TPH). It is concluded that most of hydrocarbonoclastic bacteria found in waste lubricant oil-contaminated soil are ubiquitous bacteria. These bacteria can degrade TPH in situ and potentially to be developed for bioremediation agents.

1 Introduction

Lubricant oil is an important subtances for maintaining engine performance. Lubricant oil is used to reduced friction between metal components, prevent damage caused by wear and tear, and minimize heat generated by mechanical movement [1]. Oil is derived from crude petroleum, which is extracted through a distilation process to separate different hydrocarbon fractions. This process produces long-chain hydrocarbon compounds, which are then mixed with various additives such as detergents and antioxidants to improve performance and service life [2].

Based on its composistion, oil typically contains very small amounts of plycyclic aromatic hydrocarbons (PAHs). PAHs are a group of benzene-based chemical compouns

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known to be carcinogenic and can cause lung, skin, and bladder cancer in humans with long-term exposure. The levels of PAHs and heavy metals such as lead, cadmium, and zinc in oil increase during use. Futhermore, the increasing PAHs in waste lubricant oil is also caused by oil degradation, contamination from fuel, and partial combustion occurring within the engine, making it more hazardous to health and the environment [3]. Due to its hazardous content, waste lubricant oil should not be disposed of directly into soil or water. However, not all car or motorcycle workshops have waste lubricant oil collection facilities. Small workshops with only conventional equipment often simply dispose of used oil into the environment. Our survey indicate that much of the soil around workshops has turned back and shiny, indicating that used oil is left scattered on the ground and not collected in designated areas.

Waste lubricant oil-contaminated soil typically poor in nutrients, characterized by the absence of vegetation in such areas. One effective method to address this contamination is bioremediation, which involves microorganisms such as bacteria to break down or remove harmful contaminants like PAHs from the environment. Microorganisms use PAHs as a source of carbon and energy, breaking down complex aromatic rings into smaller compounds that are easier for the environment to process [4]. The successful of the bioremediation process is determined by several environmental factors such as soil conditions, temperature, oxygen, pH, and nutrient content. In several studies, it was found that the degradation of hydrocarbon compounds also occurs optimally at a pH of 6.5–8 [5].

Previously, we successfully isolated bacteria from soil-contaminated oil and characterized several potential bacteria as bioremediation agents. Identification was conducted from various aspects, including morphological, microscopic, and biochemical analyses. The bacteria's capabilities were also tested *in vitro*, resulting in 13 potential isolates confirmed by the presence of clear zones in the medium [6]. To maximize the potential of these bacteria as biodegradation agents, it is necessary to identify the species and optimal conditions for each bacterium. The objectives of this study are to identify bacteria isolates molecularly and employ the high potential hydrocarbonoclastic bacteria and manure compost as biostimulants in *in situ* experimental study.

2 Materials and Methods

2.1 Preparation of nutrient agar (NA) medium

20 g of NA medium was mixed with 1000 ml of sterile distilled water and boiled. 6 ml of medium was put into each tube, sealed with gauze and aluminium foil. The medium was sterilised in an autoclave at 121°C and 15 psi pressure for 15 minutes. It was then cooled in a tilted position.

2.2 Preparation of mineral salt medium (MSM)

Per litre MSM medium was prepared by mixing distilled water and the following ingredients: 1,2 g NH_4Cl; 1,6 g K_2HPO_4; 0,4 g KH_2PO_4; 0,1 g NaCl; 1 g KNO_3; 20 g $MgSO_4.7H_2O$; 10 g $CaCl_2.2H_2O$; 0,05 g $FeCl_3$; 1 mL vitamin and 1 mL trace element solution. A litre of trace element solution contains the following ingredients: 50 g $MnCl_2$; 300 mg H_3BO_3; 1,1 mg $FeSO_4.7H_2O$; 190 mg $CoCl_2.6H_2O$; 2 mg $CuCl_2.2H_2O$; 24 mg $NiCl_2.6H_2O$; 18 mg $NaMoO_4.2H_2O$; 42 mg $ZnCl_2.7H_20$. The pH was adjusted to 7.3 then the medium was sterilised using an autoclave at 121^0C and 15 psi pressure for 15 minutes. 1 ml of sterile used oil was added to 1 litre of MSM medium before the medium was to be used.

2.3 Rejuvenation of Bacterial Isolates

Isolates used in this study are potential stock isolates that have been isolated from waste lubricant oil polluted soil in West Sumatra which are coded as follows: isolates A1-A5 (Bukittinggi), B1-B4 (Pasaman), C1-C4 (50 Kota), D1-D4 (Padang), and E1-E2 (Pesisir Selatan). Isolate rejuvenation was carried out on an inclined NA medium. One ose of bacterial isolate stock was streaked on the slanted NA medium and incubated at room temperature for 24-48 hours.

2.4 Cultivation of Bacteria on MSM Medium

One ose of slanted NA medium was inoculated on MSM medium. MSM medium was incubated in a shaker incubator for 4 days at 120 rpm at 37°C. Medium that had turbidity indicated the presence of growing bacteria.

2.5 Isolation of Bacterial Genomic DNA

Isolation of bacterial genomic DNA followed the procedure in the Invitorgen Genomic DNA Mini Kit (Cat no. 820-01).

2.6 Amplification of Bacterial 16S rRNA Gene

A pair of universal primers (Table 1) was used for 16S rRNA gene amplification. A total of 50 µl of PCR reaction consisted of 25 µl of dream Taq PCR, 2 µl of each forward and reverse primer, 4 µl of template DNA, and ddH_2O. PCR cycles were initial denaturation at 94°C for 3 minutes, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 48°C for 30 seconds, and elongation at 72°C for 1.5 minutes. Final elongation at 72°C for 5 minutes. Electrophoresis was performed on a 1% agarose gel for 25 minutes.

Table 1. Primers used in the amplification process of bacterial 16S rRNA ene.

No	Primers	Primer sequences	Length
1	BACTF1	5’-AGAGTTTGATCMTGGCTCAG-3’	20 bp
2	UA1B1	3’-AAGTCGTAACAASGTAACC-5’	19 bp

2.7 DNA Sequencing

Sequencing was performed at Macrogen (Singapore) with an automated DNA sequencer (ABI Prism 3100 Genetic Analyser, Applied Biosystem, USA). Sequencing results were analysed using BioEdit software and the reference sequence B. cereus ATCC 14579 (Accession No. NR 074540.1). Identification of bacterial species was carried out by aligning the 16S rRNA gene sequence results with GenBank data (NCBI) using the BLAST-N (Basic Local Alignment Search Tool-Nucleotide) programme.

2.8 Bioremediation and Biostimulation Setting

5% waste lubricant oil was added to 500g soil homogeneously, aerated for 2 days and be placed in medium sized polybags. For biostimulation, sterilised manure compost was added at different concentrations of 3%, 6%, and 9% per polybag. Furthermore, 26 ml of bacterial suspension on MSM medium was homogeneously added into the polybags. The bacterial population contained in the diluted suspension was 10^9 to 10^{11} cells/ml. The experiment was conducted for 30 days. To maintain soil moisture in the range of 17-22% and aeration, watering was done daily. The containers were aerated and humidity was maintained at 60% by adding water every day for 30 days.

2.9 TPH Degradation Measurement

The percentage of TPH degradation was measured on days 15 and 30. TPH levels were determined spectrophotometrically at a wavelength of 272 nm. A standard curve was prepared by measuring used lubricating oil at several concentrations (0.5-5 mg/L) at a wavelength of 272 nm. The relationship between the concentration of used lubricating oil and its absorbance is Y= aX+b. Y= Absorbance of the measured sample (at a wavelength of 272 nm), X= concentration of used lubricating oil in the sample. The percentage of degradation is obtained from the difference between the initial TPH concentration and the final TPH concentration.

2.10 Data Analysis

Bacterial identification data were analysed descriptively while experiment data were analysed using two-ways Anova followed by Duncan's test (DNMRT) at the 5% level.

3 Result

3.1 Amplification of 16S rRNA Gene

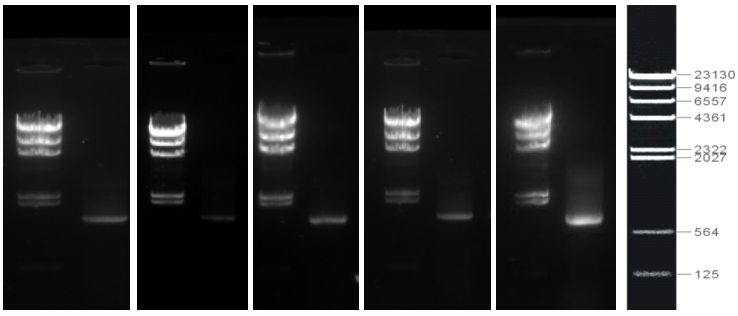

Fig. 1. Electroferogram of PCR product of 16S rRNA gene. (a) isolate A4; (b) isolate D1; (c) isolate D2; (d) isolate E1; (e) isolate E2; dan (f) λ/HindIII marker.

The representative PCR product of DNA amplification at 16S rRNA gene shown in figure 1. Amplification was carried out for thirteen isolates using BACTF1 dan UA1B1 primers. Primers amplified target gene specifically. It is revealed by DNA bands that lies between 564 bp dan 2027 bp of λ/HindIII marker. The length of amplicons is ± 1500 bp. Amplification produced thick and clear single band.

3.2 Chromatogram and Sequencing Analysis

Chromatogram of some samples showed in figure 2. Chromatograms then were alignment to the reference sequence *Bacillus cereus* ATCC 14579 (accession number NR 074540.1) in order to get the whole sequencing for bacteria identification.

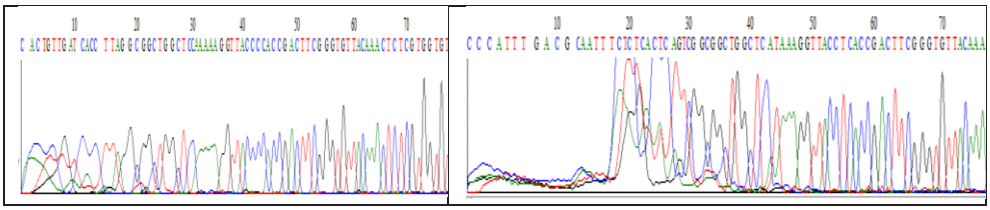

Fig. 2. Chromatogram of sample A4 (left) and D2 (right) that were aligned to reference sequence of *Bacillus cereus* as step for bacteria identifcation.

Chromatogram quite good for sequence analysis proved by single peak and clear. Every nucleotide can be read clearly and make easy to do similarity analysis using GenBank sequences and BLAST-N programme.

Table 2. The species of potential isolates.

Isolates	The Closest Strains (Accession number)	Similarity (%)	Species
A1	Alcaligenes sp	98	*Alcaligenes* sp
A2	Comamonas testosteroni strain C3 MF993019.1	99	*Comamonas testosteroni*
A4	Bacillus thuringiensis strain MSS-2 KM280648.1	99	*Bacillus thuringiensis*
A5	Bacillus amyloliquefaciens strain PPL-S7 KM226906.1	98	*Bacillus amyloliquefaciens*
B4	Bacillus thuringiensis strain S3-3 KJ496381.1	98	*Bacillus thuringiensis*
C3	Pseudomonas aeruginosa strain F2-8 KT735220.1	99	*Pseudomonas aeruginosa*
C4	Bacillus sp. DU57 HM567113.1	98	*Bacillus* sp.
D1	Bacillus sp. hb78 KF863869.1	96	*Bacillus* sp.

D2	Brevibacterium sp strain BHQ-18 KY492049.1	96	*Brevibacterium* sp
D4	Achromobacter sp. yca-a KT254215.1	99	*Achromobacter* sp
E1	Bacillus thuringiensis HF545324.1	97	*Bacillus thuringiensis*
E2	Bacillus thuringiensis J00500177.1	85	*Bacillus thuringiensis*

We recognized twelve bacteria out of thirteen isolates that have been identified. Identification of several isolates could only reveal the genus level and several isolates are probably the same species. These can be seen in isolates A1, C4, D1, C4 and D1. Therefore, more specific identification techniques are needed.

3.3 The Degradation of Total Petroleum on Day 15 and 30 of Treatment

The percentage of total petroleum hydrocarbon indicate the activities of bacteria to digest hydrocarbon contained in waste lubricant oil. In this study we measured the degradation of TPH on day 15 and 30 of treatment (table 3 and table 4).

Table 3. Total petroleum hydrocarbon level after degradation process on day-15.

Treatment groups	Compost percentages (%)			
	0	3	6	9
Negative control	16,33^{a}	23,74^{b}	23,98^{b}	23,51^{b}
Bacillus sp.	25,31^{b}	26,93^{b}	26,91^{b}	26,6^{b}
Alcaligenes sp.	24,21^{b}	24,13^{b}	25,74^{b}	25,21^{b}
Consorsium	23,23^{b}	26,05^{b}	26,34^{b}	27,32^{b}

Table 4. Total petroleum hydrocarbon level after degradation process on day-30.

Treatment groups	Compost percentages (%)			
	0	3	6	9
Negative control	18,47^{a}	26,75^{b}	26,86^{b}	28,05^{b}
Bacillus sp.	27,23^{b}	28,99^{b}	27,36^{b}	28,57^{b}
Alcaligenes sp.	26,83^{b}	29,15^{b}	28,13^{b}	31,15^{bc}
Consorsium	29,03^{b}	30,58^{bc}	31,6^{c}	31,72^{c}

4 Discussion

Molecular identification found thirteen bacterial species that capable living in soil contaminated oil. These bacterial species have the potential to degrade used oil as a source

of carbon and hydrogen for bacterial life. Oil is one of the products of petroleum processing that is rich in aliphatic and aromatic hydrocarbons. The three types of bacteria obtained are different with previous study in Japan and Iraq [7,8]. Bacteria have the ability to utilise different components in crude oil.

Bacillus is a genus of gram-positive rod-shaped bacteria and a member of the phylum Firmicutes. *Bacillus* species can be obligate aerobes or facultative anaerobes. They will test positive for the catalase enzyme when oxygen is used or present. Widely distributed in nature, *Bacillus* includes free-living species and parasitic pathogens. Under stressful environmental conditions, bacteria can produce oval endospores, which are not 'true spores' but rather structures where bacteria can reduce themselves and remain in an inactive state for extended periods. This characteristic originally defined the genus, but not all species are closely related, and many have been transferred to other genera within Firmicutes [9].

Bacillus licheniformis is a bacterium commonly found in soil. *B. licheniformis* is gram-positive and a mesophilic bacterium with optimal growth at temperatures around 50°C, but it can grow at temperatures above its optimal growth range. The optimal temperature for enzyme secretion is 37°C. When environmental conditions are unfavourable, *B. licheniformis* exists in spore form but will revert to the vegetative phase when environmental conditions improve. *Brevibacillus brevis* was formerly known as *Bacillus brevis*. *B. brevis* is a gram-positive bacterium and is aerobic. In spore form, it is commonly found in soil, air, water, and materials undergoing decomposition. *B. brevis* grows optimally at temperatures of 35–55°C, forms motile spores, and exhibits positive catalase activity, negative amylase activity, negative casein activity, positive gelatinase activity, and negative indole activity [10].

Alcaligenes eutrophus is a bacterial species capable of producing poly(3-hydroxybutyrate) (PHB), a biodegradable plastic material. This bacterium has several characteristics, including being Gram-negative, rod-shaped, non-spore-forming, growing at an optimal temperature of 30 °C, obligate aerobic (requiring O_2 to survive), facultative chemolithotrophic, and capable of producing energy by oxidising hydrogen into water. A. eutrophus is often found in soil or sediment layers containing heavy metals at high concentrations. *A. eutrophus* can synthesise PHB through the acetyl-CoA metabolic pathway, A. eutrophus can synthesise PHB through the acetyl-CoA metabolic pathway, involving three enzymes: 3-ketothiolase, acetoacetyl-CoA reductase, and PHA (polyhydroxyalkanoate) synthase [11]. Another advantage of this bacterium is its ability to utilise several aromatic compounds as the sole source of carbon and energy for survival.

The ability of microorganisms to degrade oil depends on type and concentration of oil that pollutes the environment. The biodegradation rate of the degradable component (n-alkanes) decreases as the non-degradable fraction (such as resins and asphalthenes) increases. Therefore, crude oil with heavier content is more difficult to degrade than lighter crude oil [12].

The biodegradation rate of lubricating oil contaminated soil is influenced by the type of bacteria and the percentage of contaminated soil. Bacteria used in this study are *Bacillus* sp., *Alcaligenes* sp. The percentage of polluted soil used in this study was 5%. In this study, manure biostimulant was used with a C/N ratio of 15.027. Biostimulants are useful as agents to increase degradation in polluted soils. Manure biostimulants proved be able to increase the biodegradation of polluted soil [13].

During this study, total petroleum hydrocarbon (TPH) degradation process was observed on day 15 and day 30. In table 3, it can be seen that the bacterial consortium (*Bacillus* sp. and *Alcaligenes* sp.) added with 9% biostimulant showed the highest degradation ability on day 15 . In accordance, on day 30 the bacterial consortium (*Bacillus* sp.1 and *Alcaligenes* sp.1) with 9% biostimulant also showed the highest degradation ability to degrade TPH in the used lubricating oil-contaminated soil. However, the ability of homogenous bacteria on day 30 is different from day 15. On day 15, *Bacillus* sp.1 show the highest ability to degrade TPH

while on day 30 *Alcaligenes* sp.1. This indicated that the activity of *Alcaligenes* sp. and *Bacillus* sp. will be better in degrading TPH when they are in consorsium. The bacterial consortium has a higher degradation ability because the consortium is a group of microorganism that works together. A group or consortium of microorganisms is needed and will naturally occur, because synergistically they have more ability to degrade an organic compound. This means that it increases the possibility of obtaining energy and surviving [14].

The results of further tests on the effect of the amount of biostimulants on the percentage of TPH degradation in Table 3 show that there is no significant difference between the three treatments given (3%, 6%, and 9% biostimulants). However, there was a significant difference between the treatment and negative control. This condition is slighly different on day 30 observations. Consorsium bacteria have the highest biodegradation on day 30.

From this study can be concluded that most of hydrocarbonoclastic bacteria found in waste lubricant oil-contaminated soil are ubiquitous bacteria. These bacteria can degrade TPH *in situ* and potentially to be developed for bioremediation agents.

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