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Isolation and Identification of Hydrocarbon Degradation Bacteria and Phosphate Solubilizing Bacteria in Oil Contaminated Soil in Bojonegoro, East Java, Indonesia

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ABSTRACT

Petroleum is a mixture of hydrocarbon complexes with organic compounds from sulfur, oxygen, nitrogen, and metal-containing compounds. These organic compounds can be used as substrate for bacterial growth. This study aimed to isolate and identify hydrocarbon degrading bacteria and phosphate solubilizing bacteria in oil-contaminated soil in Bojonegoro. This study used an exploration method to find each of the two types of hydrocarbon degrading bacteria and phosphate solubilizing bacteria from soil samples in Bojonegoro that contaminated by oil. Identification of isolates bacterial included macroscopic observations of bacteria, gram staining on bacterial cells and physiological tests. Macroscopic observations include the form of colonies, colony diameter, colony color, colony edge, and elevation. The physiological test using Microbact Identification System to determine the physiological characteristics of bacteria so that genera and types of bacteria can be known. The identification of organisms was based on changes in pH and use of the substrate. The results of data analysis were obtained from five types of bacteria from soil samples that contaminated by oil which were successfully isolated. After identification of species was done, four species of bacteria were obtained, namely *Pseudomonas pseudomallei, Pseudomonas fluorescens-25, Flavobacterium odoratum,* and *Enterococcus sp.*

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1. INTRODUCTION

Petroleum is consist of hydrocarbon complexes with organic compounds such as sulfur, oxygen, nitrogen and metal elements which include Cd,Al, As, Hg, Ni, Cr, Cu, Pb, Zn, Se, and some radionuclides (Ogbo and Okhuoya, 2011). The main materials contained in petroleum are parafinhydrocarbon compounds, saturated alicyclic hydrocarbons, and aromatic hydrocarbons that are carcinogenic and organic pollutants (Santhini *et al.,* 2009). Petroleum can be categorized into four classes, namely: aromatic, saturated, asphaltene (fatty acids, phenols, esters ketones, and porifrin), and resins (quinolin, pyridine, carbasol, amide, and sulfoxide). The hydrocarbons type and class influence the process of petroleum biodegradation (Das and Chandran, 2011).

Petroleum contaminated environment and its derivatives is a common problem found in petroleum mining site area. Oil that spills into soil resulting from petroleum mining activities cause contamination in soil. The contamination will make soil having very low minerals nutrient content and very high hydrocarbon compounds (Jain *et al.,* 2011). The ex-mining land needs to be processed first to eliminate the hydrocarbon content before it is used as growth media for plant with efficient technology, namely by bioremediation. Bioremediation is an effective technology for transforming toxic components into less toxic products through the activity of microorganisms, so it does not have a negative impact on the environment (Millioli *et al.,* 2009; Karigar and Rao, 2011).

Microorganisms such as bacteria can be used in the bioremediation process to break down certain compounds that contained in petroleum. Some types of bacteria that have these abilities were *Pseudomonas, Butkholderia, Agrobactericum, Sphingomonas, Rhodococcus, Flavobacterium, Mycobacterium,* and *Bacillus* (Haritash and Kaushik, 2009; Kafilzadeh *et al.,* 2011; Nandiyanto *et al.,* 2016). Bacteria has an enzymatic capacity to associate with hydrocarbon, making it effective to use in the degradation process of hydrocarbon complexes in oil-contaminated environments. The increase of organic acids is usually followed by a decrease in pH, resulting in dissolution of P bound by Ca. Some of these low molecular weight organic acids are reported to reduce Al interchangeable toxicity, including Fe, Mn, and Cu absorbed by corn planted on acid soil so that they are at a normal level.

Meanwhile, hydrocarbons in the environment can be biodegradable, especially by bacteria and fungi. In a terrestrial and aquatic ecosystem, the fraction of the total heterotropic community represented by bacteria and fungi using hydrocarbons was varies greatly, around 6-82% for soil fungi, 0.13-50% for soil bacteria, and 0.003-100% for marine bacteria. The bacteria that play the most important role in degrading hydrocarbons in the marine and soil environments are *Achromobacter, Flavobacterium, Alcaligenes, Arthrobacter, Achinobacter, Nocardia, Pseudomonas* sp., *and Coryneferum*and *Bacillus* (Van Hamme *et al.,* 2003). Other researchers prove that some types of microorganisms are able to use sources of carbon to reforming hydrocarbons process including: *Bacillus, Pseudomonas, Acinetobacter, Alcaligenes, Xanthomonas, Benecdea, Brevibacterium, Methylococcus, Methvlobacterium,* and *Mycobacterium* (Mrozik *et al.,* 2003). In Kafilzadeh's research (Kafilzadeh *et al.,* 2011), it was stated that there are 10 genera of bacteria that are best used for hydrocarbon degradation. The genus includes: *Bacillus, Streptococcus, Staphylococcus, Corynebacterium, Shigella, Acinetobacter, Alcaligenes, Klebsiella, Enterobacter,* and *Escherichia*. Among other bacteria, Bacillus was the best hydrocarbon degrading bacteria.

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Given the nature of certain bacteria and fungi that are beneficial to increase the phosphate availability in the soil and capable of degrading hydrocarbons in oil-contaminated soils, this type of microbes can be used in an effort to find oil-contaminated bioremidiation models. Phosphates that are widely available to plants will make plant growth and resilience even better. More degraded hydrocarbons will produce minerals or nutrients that important for plant growth.

The creation of environmental conditions is suitable for microorganisms such as temperature, pH, salinity, organic matter and soil moisture, and nutrients is very important to support the activity of microorganisms that degrade hydrocarbons. Therefore, measuring the speed of biodegradation with indigenous microorganisms is the first step in the role of microbiology in the soil bioremediation process. Therefore, bioremediation can be done by using indigenous microorganisms under optimum conditions or by inoculating non-indigenous microorganisms if needed. However, bioremediation using indigenous microorganisms has a positive effect considering that the microorganisms used are selected microorganisms that have been adapted to the exposed environment (Hamamura *et al.*, 2006), which in this study is an environment exposed to oil. Kabirun (2004) also revealed that the mycorrhizae with the same species but originating from different environments would have different effects in terms of the effectiveness of increasing P nutrient availability. This shows that there is genetic and physiological diversity in the population of soil microorganisms originating from the different sources. Some theoretical and empirical studies in the form of supporting research results indicate that indigineous microorganisms are microorganisms that have better effectiveness than non-indigestous microorganisms.

This purpose of this study was to isolate and identify the hydrocarbon degradation bacteria and phosphate solubilizing bacteria in oil-contaminated soils. This research was a preliminary study in examining the patterns of interaction between hydrocarbon degrading bacteria, phosphate solubilizing bacteria, rhizobium bacteria and mycorrhizal fungi, through soybean test plants which are often found in Bojonegoro soil that contaminated by oil. The results of this study are expected to be used to compile a bioremidiation model on oil contaminated soils using the benefits derived from the pattern of multisymbiotic relationships between organisms and the role of each symbiont in the relationship pattern, so that plants test could survive in oil-contaminated soil.

2. MATERIALS AND METHODS

The initial stage of this research was carried out exploratively which aimed to find phosphate solubilizing and hydrocarbon degradation bacteria in oil-contaminated soil in Bojonegoro. The study was carried out by isolating bacteria at several points in soil sampling. All bacteria that have been isolated are then identified.

The tools used in this study include: Sample bottles, incubators, refrigerators, analytic balance, scales, light microscopes, spirits lamps, alarm cups, measuring cups, enlemeyer bottles, petri dishes, test tubes, test tube racks, pipettes, stirring rods , needle ose, glass object, pH meter, thermometer.

The materials used in this study were: Soil samples taken from oil-contaminated soil in Bojonegoro. KNA Media (Nutrient Agar Broth). Gram staining material consists of: violet crystals, iodine, Ethyl alcohol 95%, Safranine. Modified media of Czapex without

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sucrose, spirits, aquades, condominium foil, cotton, label paper, filter paper, and tissue.

2.1. Initial Isolation of Soil Samples

Bacteria were isolated from soil that contaminated by oil from the area around the oil mining site in Bojonegoro. 10 grams of soil sample was put into 90 ml of sterile distilled water, and homogenized. Then 1 ml was taken and put in a test tube containing Czapex Broth without sucrose, and the sample was incubated at 300C for 7 days, samples incubated for 7 days were inoculated using a pour method (Pour Plate Method) of 1 ml into petri dishes containing KNA media and incubated at 300C. Growing bacteria are inoculated in a test tube containing KNA media until pure isolates are obtained. The bacterial colonies that were grown were observed morphologically, gram staining and physiological tests.

2.2. Identification of Isolates

Identification of bacterial isolates included macroscopic observations of bacterial isolates, gram staining on bacterial cells and physiological tests. Macroscopic observation of bacterial isolate colonies include: 1. Colony shape, 2. Colony diameter, 3. Colony color, 4. Colony edge, 5. Elevation. Gram staining is a multilevel staining, namely painting that uses more than two types of substances and is done in stages to determine the shape and type of gram bacteria.

Physiological tests with Microbact Identification System are used to determine the physiological characteristics of gram negative bacteria, so that genera and types of bacteria are known. The format is in the form of a simple test strip or micro-plate and the results are clearly seen as different color reactions that can be interpreted using Microbact. Each kit consists of 12 (12A, 12B) or 24 (24E) miniature biochemical tests. The identification of organisms is based on changes in pH and substrate use.

Identification of gram-positive bacteria uses the book Bergey's Manual of Determinative Bacteriology Eighth Edition and Cowan And Steel's Manual For The Identification of Medical Bacteria Third Edition, while for identification of bacteria obtained with bacteria that actually use similarity coefficient formulas namely:

$$\text{Coefficient of similarity} = \frac{A}{A+B+C} \text{x } 100\%$$

Remarks: A: Positive characteristics for both lines. B: Positive characteristics of line one, negative characteristic of line two. C: Negative characteristics of line one, positive characteristic of line two. Data analysis in this study was carried out descriptively.

3. RESULTS

3.1. Data On Chemical And Physical Analysis Of Oil-Polluted Soils

3.1.1. Analysis of TPH and N, P, K, C / N ratio of oil-contaminated soil ratio

Soil samples used to determine the chemical properties of oil-contaminated soils were taken from the area around modern oil mining and traditional oil mining in Wonocolo, Beji, and Harjomulyo Villages, Kadewan District, Bojonegoro Regency. The results of analysis of soil samples on the chemical properties obtained as shown in **Table 1**.

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Table 1. Analysis of Chemical Properties of Soil in Oil-contaminated Soil Samples

No	Parameter	Value (unit)	Comparative Content for Criteria for assessing soil chemical properties
1	TPH	41.200 mg/kg	Very high
2	Nitrogen (N)	0,20%	Low<0,21
3	Phosphat (P)	0,01%	Very low< 10
4	Kalium (K)	0,22%	Low (0,1-0,2)
5	Carbon (C)	8,53%	Very high> 4
6	C/N ratio	42,7	Very high (>15)

The data in the table shows that TPH and C levels are very high in category, therefore the C / N ratio is also high. Meanwhile the nutrient content (N, P, and K) still shows the low or very low category. To determine contamination oil in soil, TPH parameter is used. TPH level is used for quantifying contamination in environmental that originated by various petroleum hydrocarbon (PHC) products such as oils,fuels, waxes, lubricants, and others. TPH is a complex compound consisting of aromatics, alkane, sulfur, nitrogen, and asphaltic fractions (Sood *et al.,* 2010).

3.3.2. Environmental Physics Properties Around Sampling of Soil

The results of measuring the environmental conditions around the location of soil sampling are presented in **Table 2**.

Table 2. Results of Measurement of Soil Environmental Conditions

No	Parameter	Measurement results
1	Air temperature	36 ^{o}C
2	Humidity	40%
3	pH	5.8
4	Soil temperature	39 ^{o}C

3.2. Results Of Bacterial Isolation From Oil-Polluted Soil Samples

3.2.1. Characteristics of Bacterial Isolates found in Oil-Polluted Soil in Bojonegoro

Bacterial isolates were characterized based on observation of colony morphology, gram staining, physiological characteristics through biochemical tests using Microbact Identification System. The bacterial gram test results were matched with the book Cowan and Steel's Manual for The Identification of Medical Bacteria and the Bergey Manual of Determinative Bacteriology, Eighth. Bacteria with code isolates B1 and B2 derived from soil samples 1 and bacterial isolates with code A1, A2, and A3 derived from soil samples 2. Identification of these bacteria was based on observations of colony morphology and gram staining, the results of which are shown in **Table 3**.

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Table 3. Morphology of Bacterial Colonies in Oil-contaminated Soils

Macroscopic Observation of Bacterial Colonies							
Isolate	**Shape**	**Diameter**	**Colour**	**Edge**	**Elevation**	**Internal Structure**	**Gram staining**
B1	Irregular and diffuse	0,5-1 cm	Yellow	Slippery / even	Convex	Not translu-cent	Gram negative, bacil
B2	Round	2-4 mm	Milky white	Slippery / even	Low convex	Coarse grained	Gram negative, bacil
A1	Round	0,5-1 mm	Red	Slippery / even	Flat	Not translucent	Gram negative, bacil
A2	Irregular and diffuse	0,5-1 cm	Yellow	Slippery / even	Convex	Not translucent	Gram negative, shortbacil not sporadic
A3	Irregular and diffuse	1-2 cm	White	Slippery / even	Convex	Not translucent	Gram positive, coccus

B1 : *Pseudomonas pseudomallei*; B2 : *Pseudomonas fluorescens-25*
A1 : *Flavobacterium odoratum*; A2 : *Pseudomonas pseudomallei*
A3 : *Enterococcus sp.*

Gram staining results showed that isolates B1, B2, A1, and A2 were gram negative bacteria, while A3 isolates were gram positive bacteria. The basis for distinguishing bacteria in gram positive or negative groups is the result of the staining of the four reagents used in staining. Giving the basic color of the crystal violet will cause all cells to form the CV-I complex (Crystal violet-Iodine) which will bind to the Mg-RNA component of the cell wall, forming an alcohol-insoluble Mg-RNA-CV-I complex. Lugol in the test was used as a reinforcement solution for the Mg-RNA-CV-I complex. Alcohol (95%) as a decolorization compound will dissolve fat. Gram (+) bacteria have a small fat content, so that when washing with alcohol, fat will dissolve and form small pores which are then covered by dehydrated proteins that cause closed pores. As a result, the primary dye is difficult to wash and the cell becomes purplish blue. The fat in gram (-) cells is quite large, so that the time of alcohol dissolution produces large pores which cannot be covered by dehydrated pores. Consequently, alcohol washes all Mg-RNA-CV-I complexes and color loss cells. The fourth reagent in coloring is safranin which is used to replace the basic color that has been lost because of the washing of alcohol. The results are, gram (-) bacteria will be red and gram (+) bacteria remain purple (Beveridge, 2001).

The bacterial characterization carried out was then followed by a series of physiological tests to identify bacterial species. Physiological tests conducted showed that there were 4 species of bacteria from 5 bacterial isolates that had been isolated from oil-contaminated soil, as shown in **Table 4**.

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Table 4. Microbact Identification System Results of Bacterial Isolates from Oil-contaminated Soil

No	Bacterial Isolate Code	Code Microbact 2000	Identification Results	
			Name of Species	Percentage of Probability
1	B1 (gram negative)	767563765	*Pseudomonas pseudomallei*	99,99%
2	B2 (gram negative)	605130321	*Pseudomonas fluorescens-25*	98,56%
3	A1 (gram negative)	600100000	*Flavobacterium odoratum*	66,31%
4	A2 (gram negative)	605520371	*Pseudomonas pseudomallei*	96,15%
5	A3 (gram positive)	-	*Enterococcus sp.*	75%

The physiological test results using Microbact Identification System showed that bacterial isolates B2, A1, and A3 came from different species, while isolates B1 and A2 were from the same species, namely *Pseudomonas pseudomallei.*

3.2.2. Characterization of Indigenous Bacteria in Oil-contaminated Soil

Bacterial identification based on biochemical activity tests is done by comparing the biochemical activity of each bacterium. The biochemical activity of each bacterium is different because each bacterium has a different enzymatic activity. **Table 5** shows the bio-chemical /physiological test data of bacteria found in oil-contaminated soils.

Physiological characteristics include the nature and ability of bacteria to grow in the media (Lysine, Ornithine, Arginine, ONPG, indole, TDA, Gelatin, Malonate-amino acids) H2S, citrate, Urease, VP, (Mannitol,Glucose, ONPG, Xylose, Sorbitol, Inositol, Rham-nose, Lactose, Sucrose, Adonitol, Raffinose, Arabinose, Salicin-carbohydrates). In the isolation of bacteria B1, *Bukholderia pseudomallei* (*Pseudomonas pseudomallei*) undergoes a positive reaction when tested on glucose, xylose, Urease, V-P, Citrate, Malonate, Rhamnose, Lactose, Arabinose, Adonitol. Isolate 1 has no reaction when tested on Lysine, Ornithine, H2S, Mannitol, Indole, TDA, Gelatin, Sorbitol, Sucrose, Raffinose, Salicin, Arginine. Isolate 1 is a positive, motile and can reduce nitrate bacteria with oxidase.

Isolate B1, which is *Pseudomonas pseudomallei*, a positive, motile and nitrate-reducing oxidase bacterium, experiences a negative reaction to the H_2S formation test, including in the Indole test, Thryptophan deaminase, gelatin, Adonitol and Salicin. The iso-lat shows a positive reaction when tested on Lysine, Ornithine, acids from glucose, Mannitol, and Xylose. Positive tests were also shown on ONPG, urea hydrolysis, citrate use, malonic inhibition, and acid formation reactions from Inositol, Sorbitol, Sucrose, Lactose, Arabinose, Raffi-nose, and Arginine dehydrolase.

Isolate B2, *Pseudomonas fluorescens* is a positive, motile oxidase bacterium and cannot reduce nitrate. This bacterium experiences a negative reaction to the formation of Lysine dehydrogenase, Ornithin decarboxylacil, H_2S production, including acid test from Mannitol, INPG, Indole Gelatin, malonate inhibition, acid formation from Inositol and acid from sorbitol, and acid formation from Adonitol, Raffinosa and Salicin. The bac-terial isolate also shows a positive

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reaction when tested on acids from glucose, xylose, hydrolysis of urea, use of citrate, thryptophan deaminase, acids from Sorbitol and Arabinose and Arginine dehydrolase.

Isolate A1, *Flavobacterium odoratum*, is a positive, motile oxidase bacterium and cannot reduce nitrate. This bacterium shows a positive response to the urea hydrolysis test. Meanwhile, from a series of other physiological tests that are presented, it shows some negative reactions.

Isolate A2, *Pseudomonas pseudomallei* is a positive, motile oxidase bacteria and cannot reduce nitrate. This bacterium experiences a negative reaction to the formation of lysine dehydrogenase, decarboxylacyl ornithin, H2S production, including acid test from mannitol, indole gelatin, tryptophan deaminase, gelatin test, malonic inhibition, acid from inositol and acid from sorbitol, and Raffinosa acid Salicin. Different results were shown by isolates of the bacteria, which showed a positive reaction when tested on acids from glucose, xylose, ONPG, hydrolysis of urea, use of citrate, acids from sorbitol, sucrose, lactose, arabinose, adonitol and Arginine dehydrolase.

Isolate A3, *Enterococcus* sp. is a positive, motile and can reduce nitrate bacteria with oxidase. This bacterium experiences a negative reaction in the H_2S production test, Indole reaction, tryptophan deaminase, acid from inositol, acid from laktose, Odonitol and Salicin, while for other physiological tests it shows a positive reaction.

4. DISCUSSION

4.1. Results of Analysis of Chemical and Physical Properties of Oil-Polluted Soils

Based on the data in **Table 1** shows that the TPH content of oil-polluted soil used in this study is still high at 41,200 mg/kg, while soil N and K levels including low with available P-levels on the soil are in the very low category. Meanwhile C levels are very high, so the C / N ratio obtained is also very high.

The condition of oil contaminated soil is in accordance with the properties found in petroleum which is a mixture of hydrocarbon complexes with organic compounds from oxygen, sulphur, nitrogen and metal elements, especially iron, nickel, and copper (Harayama *et al.* 1999) . The main materials contained in petroleum are aliphatic hydrocarbon compounds, aromatic hydrocarbons, and alicyclic hydrocarbons. In addition, petroleum also contains oxygen, nitrogen, carbon, sulfur, and other elements that have varied physical and chemical properties (Liu and Kujawinski, 2015). The content of some of these elements causes high levels of C and TPH in petroleum contaminated soils. Considering that TPH is a measurement of the concentration of hydrocarbon pollutants of oil in the soil or the weight of all oil hydrocarbon pollutants in soil samples which are often expressed in units of mg hydrocarbons/kg of soil. These include the availability of N, P, and K that are absorbed in oil elements and contested hydrocarbon compounds (Romanus *et al.* 2015).

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Table 5. Biochemical Test (Physiology) of Bacterial Isolates from Oil-Polluted Soil

Physiology Test	Reaction Index	Bacterial Samples									
		B1		B2		A1		A2		A3	
		Result	Octal code	Result	Octal Code	Result	Octal code	Result	Octal code	Result	Octal code
Oxidase	4	+		+		+		+		+	
Mortility	2	+		+		+		+		+	
Nitrate	1	+		-		-		-		+	
Lysine	4	+		-		-		-		+	
Ornithine	2	+		-		-		-		+	
H_2S	1	-		-		-		-		-	
Glucose	4	+		+		-		+		+	
Mannitol	2	+		-		-		-		+	
Xylose	1	+		+		-		+		+	
ONPG	4	+		-		-		+		+	
Indole	2	-		-		-		-		-	
Urease	1	+		+		+		+		+	
VP	4	+		-		-		-		+	
Citrate	2	+		+		-		+		+	
TDA	1	-		+		-		-		-	
Gelatin	4	-		-		-		-		-	
Malonate	2	+		-		-		-		+	
Inositol	1	+		-		-		-		-	
Sorbitol	4	+		-		-		-		+	
Rhamnose	2	+		+		-		+		+	
Sucrose	1	+		+		-		+		+	
Lactose	4	+		-		-		+		-	
Arabhi-nose	2	+		+		-		+		+	
Adonitol	1	-		-		-		+		-	
Raffinose	4	+		-		-		-		+	
Salicin	2	-		-		-		-		-	
arginine e	1	+		+		-		+		+	

B1 : *Pseudomonas pseudomallei*; B2 : *Pseudomonas fluorescens-25*
A1 : *Flavobacterium odoratum;* A2 : *Pseudomonas pseudomallei*
A3 : *Enterococcus sp.*

4.2. Results of Isolation of Indigenous Bacterial Species of Oil-Polluted Soil

The bacterial isolates that were successfully isolated and identified in oil-polluted soils were about five bacterial isolates. However, out of the five bacterial isolates, there were two bacteria that had the same species. Therefore, the bacterial species in this study which were successfully isolated and identified were only four species of bacteria. These bacteria are *Pseudomonas pseudomallei, Pseudomonas fluorescens-25, Flavobacterium odoratum, and Enterococcus sp.*

Organic compounds that contaminated the environtmen are able to insert into cytoplasm membrane and affect functions of physiological membrane. Bacteria had to adapt some mechanisms to survive from the

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damage from toxic compounds contaminants and to prevent toxic compounds to be accumulated in cell. Adaptation of cell maintains the status of membrane fluidity and ratio between non bilayer or bilayer phospholipids as well as the efflux of toxic compounds, mechanisms of protein repair, and degradation of toxic contaminants. Low energy that consumed by cell that evolve in adaptation is required for other physiological functions. Bacteria that have an ability to survive in toxic environment could be used as an agent that clean contaminated areas through bioremediation technologies (Murinova and Dercova, 2014).

Bioremediation methods by utilizing bacteria require a consideration of the physical and chemical properties of the environment in which bacteria originate such as pH, salinity, pressure, temperature, and nutrient availability (Das and Chandran, 2011). Only bacteria considered can increase their role in improving oil contamination. Fungi, yeasts, algae and protozoa are apparently not suitable for soil bioremediation because of the size and inability of organisms to grow under existing conditions, especially in the reservoir for remediation purposes, so that only bacteria are suitable for the bioremediation process. Many contaminated soils have high NaCl concentrations and require handling using bacteria that are tolerant to these conditions. Biosurfactant and polymer-producing bacteria can grow at NaCl concentrations to 8% and selectively produce "walls" to deal with existing oils (Millioli *et al.,* 2009).

The microbial success in degrading hydrocarbons (oil) is very much dependent on their ability to break down carbon which is in relatively high temperatures (70 to 90 ° C), including pressure (2,000 to 2,500 lb/in 2) and media quality (1.3 to 2.5%) which allows the bacteria to continue growing. Therefore, thermophilic bacterial isolates have good potential to be used in the environment, even anaerobic thermophilic bacteria that has the ability to grow at temperatures of 80 to 110°C have been successfully isolated and cultured. All of these organisms live autotrophically on S, H and CO_2 by the process of metagenesis and heterotrof depend on organic substrate by respiration or by anaerobic fermentation (Madihah *et. al.,* 1998).

Thus it can be said that the four bacterial species that were isolated from oil-polluted soil were indigenous bacterial species which have good adaptive power to oil contaminated soil conditions in Bojonegoro.

In addition to mycorrhizae, organisms capable of increasing P availability are phosphate solubilizing bacteria. These bacteria can dissolve P which is insoluble, so it has a form available to plants by dissolving it with organic acids. Some types of bacteria and fungi that have this ability include: *Pseudomonas, Mycobacterium, Micrococcus, Flavobacterium, Penicillium, Sclerotium, Bacillus.* These microbes have the ability to produce biosurfactants consisting of various chemical structures such as fatty acids, polysaccharides, peptides, glycolipids, and hydrophobic and hydrophilic moetie proteins which can reduce surface pressure and interphase between individual molecules. This makes microbes have the potential in the process of emulsification and increases the process of oil neutralization (Borah and Yadav, 2016). The microbes in their activities also have the ability to produce organic acids which will be followed by a decrease in pH, so that P is bound by Ca to dissolve. In addition to increasing P availability, some low-molecular organic acids can also reduce Al exchangeable toxicity, including reducing

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Fe, Mn and Cu absorbed by maize planted on acid so that the levels are normal.

Based on the results of previous studies, it was shown that the bacterial isolates that were identified were soil bacteria species that have the ability to dissolve phosphate. Since these bacteria are isolated from oil-contaminated soil, this also indicates that these bacteria also have the ability to degrade hydrocarbons which are relatively high in oil-contaminated soils that are used as indicated by TPH values and C which are high in the test soil samples (**Table 1**). This can be understood because bacteria will fulfill their energy needs to grow and relax their metabolic activities by using sources in their environment. In the context of this study, they are the utilization of hydrocarbons which are relatively high in the environment by the four bacteria.

The mechanism of bacteria in conducting phosphate is by changing the status of P availability to the form available to plants. Soil microorganisms affect the solubility of phosphate compounds through the secretion of organic acids that produce inorganic phosphate and inorganic phosphate mobilization for their own microorganism needs. The role carried out by microorganisms is to transform phosphates including by 1) dissolving inorganic P compounds can occur through the production of some organic acids, 2) mineralization of organic compounds by freeing inorganic phosphates. In this case, soil microorganisms synthesize the enzyme which act as a biocatalyst in the hydrolysis reaction of P-organic which produces P-inorganic, 3) Mobility of inorganic P into microbial cells. This is because phosphorus is needed by soil microorganisms not only as a constituent material, but also as energy source for all metabolic activities in cells (Rathi and Gaur, 2016).

The mechanism of dissolution through chelation between acids produced by isolates and cations that bind phosphate, so that the size of phosphate dissolution depends on the affinity of acids in binding to phosphate binding cations. Acid affinity is determined by the type of acid, so the size of phosphate dissolution by bacteria depends not only on acid pH, but also on the type and amount of acid produced (Vinas *et al.,* 2005).

Based on the ability of bacterial isolates found in degrading hydrocarbons, it shows that petroleum biodegradation is a natural process, which involves microorganisms that can transform and decompose petroleum hydrocarbons into other simpler components (Xenia and Refugio, 2016; Singh *et al.,* 2014). The mechanism of transformation and decomposition carried out by microorganisms is one example of the breakdown of n-alkane hydrocarbons. The breakdown of the n-alkane hydrocarbon molecule by microorganisms is initiated by a multi-complex enzyme monooxygenase (ω-hydroxylase) system that can oxidize alkanes into primary alcohols. Then the primary alcohols formed are oxidized to aldehyde compounds and eventually become fatty acids. The resulting fatty acids can be directly broken down into CO_2 through the a-oxidation process or used as nutrients (carbon sources and energy) for cell growth through the process of β-oxidation (Holst *et al.,* 2007; Das and Chandran, 2011).

In other research found that at low temperatures, the oil viscosity was increased while the toxic volatility of the low molecular weight hydrocarbons were reduced, delaying the onset of biodegradation (Atlas, 1975; Das and Chandran, 2011)

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Bacteria in their life activities require carbon molecules as a source of nutrition and energy to metabolize and reproduce. In particular, groups of microorganisms capable of using carbon sources derived from hydrocarbons are called hydrocarbonoclastic microorganisms (Harayama *et al.,* 1999).

Most marine environments consist of bacteria that involve in hydrocarbon degradation with different metabolic capabilities. These bacteria are categorized based on the carbon source used, as "obligate hydrocarbonoclastic" when they can grow only with a specific carbon source or "facultative hydrocarbonoclastic" when they are able to grow with alternative carbon sources (Harayama *et al.,* 1999)

The most important genus of bacteria using hydrocarbons based on their isolation frequency are *Achromobacter, Acinetobacter, Aeromonas, Corynebacterium, Flavobacterium, Methylobacter, Methvlobacterium, Pseudomonas* (Rodrigues *et al.,* 2015).

The characteristics of hydrocarbon chlorlastic microorganisms that are not possessed by other microorganisms are their ability to express hydroxylase enzymes, namely hydrocarbon oxidizing enzymes, such as alcohol dehydrogenase and alkane hydroxylase which play a role in the biodegradation of hydrocarbon (Parthipan *et al.,* 2017).

5. CONCLUSION

The bacterial isolates that were successfully isolated and identified in oil contaminated soils were five bacterial isolates. However, out of the five bacterial isolates, there were two bacteria that had the same species, namely *Pseudomonas pseudomallei.* Therefore, in this study, four species of bacteria were isolated and identified, namely *Pseudomonas pseudomallei, Pseudomonas fluorescens-25, Flavobacterium odoratum, and Enterococcus sp.* These bacteria are bacteria that have the ability to dissolve phosphates and simultaneously degrade hydrocarbons.

6. AUTHORS' NOTE

The authors declares that there is no conflict of interest regarding the publication of this article. Authors confirmed that the data and the paper are free of plagiarism.

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