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ISOLATION AND IDENTIFICATION OF PHOSPHATE SOLUBILIZING BACTERIA AND HYDROCARBON DEGRADATION BACTERIA IN LAPINDO MUD SIDOARJO - EAST JAVA - INDONESIA

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Abstract

Lapindo mud, the biggest mud disaster in Sidoarjo, Indonesia since 2006, still offers numbers of constituents such as heavy metals and crude oil. The oil is produced by a mixture of complex hydrocarbon with organic compounds from sulfure, oxygen, nitrogen and metal. Moreover, those organic compounds can be used as substrates for bacteria growth. Thus, this paper aims at isolating and identifying potential bacteria in dissolving phosphate and degrading hydrocarbon in Lapindo mud. The characterization of phosphate solubilizing bacteria is obtained by using Pikovskaya media, in which a clear zone indicating potential phosphate solubilizing bacteria. The bacteria identification includes macroscopic observation in a bacteria colony, gram-coloring bacteria and physiological tests. Under descriptive analysis, results show that there are three species namely *Pseudomonas pseudomallei*, *Pseudomonas fluorescens-35*, and *Pseudomonas stutzeri* with the percentages of probability 99.99%; 58.26% and 66.40% respectively.

Keywords: Hydrocarbon degradation bacteria, Isolation and identification, Lapindo, Phosphate solubilizing bacteria.

1. Introduction

An unexpected mud was erupted and fluided on 29 May 2006 as a result of too deep hydrocarbon exploration in Porong, Sidoarjo, East Java, Indonesia. Up to this day, the eruption site is well known as Lusi (abbreviation of *Lumpur*/mud-Sidoarjo). In addition to the disaster area, the 100°C mud and gas covered more than 6.3 km^2 of land in May 2007 spreading between Sidoarjo and other nearby villages in the north. Consequently, thousand people have been evacuated due to the hazardous mud flood. The Lusi samples collected in 2006 contain thermogenic hydrocarbons mixed with a minor microbial CH_4 component [1, 2].

In addition, the Lusi flow has resulted of the loss of vegetation, flora and fauna, yet potentially contaminating surface water and groundwater and when the mud extends, micro-climate changes will occur [1, 2]. Previous finding shows that there are no environmental effects of heavy metals (Mn, Zn, Cu, Cr, Cd, Pb, Co, Ni, Hg, and As) caused by the mud, unless when the metals are associated with other elements. In contrast, the physical and chemical mud-water was above the environmental standard [3]. Nevertheless, the Lusi is a potential to be used even some prerequisites need prior processes. One alternative is the bioremediation using multisymbiotic interaction between phosphate solubilizing bacteria, hydrocarbon degrading bacteria, rhizobial bacteria and mycorrhiza with legume as a tested plant in the concepts of bioremediation and remediation of the structure and texture of the Lusi before its usage as a medium for plants due to the high content of Total Petroleum Hydrocarbon (TPH) and heavy metals.

Bioremediation can be defined as a process using microorganisms or their enzymes to return the contaminated environment from its original condition. It can be employed to attack specific contaminants, such as oil spills that are broken down using multiple techniques including the addition of fertilizer to facilitate the decomposition of crude oil by bacteria [4].

A previous study on bioremediation of crude oil contaminated soil in Bojonegoro, East Java, Indonesia, indicated that there was a positive effect of the interaction between hydrocarbon degrading bacteria, phosphate solubilizing bacteria, Rhizobium bacteria and Mycorrhizal to decrease TPH and to increase of N, P, C/N ratio, also the percentage of mycorrhizal infection and the percentage of effective root nodules as well as to increase plant growth on oil-contaminated soil in Bojonegoro [5].

Phosphate solubilizing bacteria are able to solubilize phosphate and made it available for plants by solubilizing the organic acids. Some types of bacteria and fungi that have this ability include *Pseudomonas, Mycobacterium, Micrococcus, Flavobacterium, Penicillium, Sclerotium, Bacillus* and *Thiobacillus* bacteria and *Nitrosomonas* [6]. Meanwhile, the hydrocarbon compounds in the environment can be biodegraded by bacteria and fungi [7]. This research aims at isolating and identifying potential bacteria in solubilizing phosphate and degrading hydrocarbon compounds from the Lusi. Both identified phosphate solubilizing and hydrocarbon degrading bacteria are then used for the Lusi bioremediation process along with Rhizobium and Mycorrhiza that use legume as a tested plant. Through the process of biodegradation contaminant compounds in soil, it will be enhanced by the presence of vegetation that is effective at a depth of 0-20 cm, including the quality of Rhizodeposisi highly dependent on the existing vegetation [8].

2. Materials and Methods

At this stage, exploratory study was conducted to find out phosphate solubilizing bacteria and hydrocarbon degrading bacteria in Lusi. The study was conducted by isolating the bacteria at some point soil samplings. All bacteria were isolated and then identified through morphology test, staining gram and physiology test. The initial isolation from soil samples several bacteria isolated from Lusi. These isolations were obtained by isolating the Lusi samples using the pour plate method. A total of 10 grams of Lusi samples included in 90 mL of medium Atlas Oil Surface Agar (AOA) then diluted using serial dilutions until the 10^7. The results of each dilution were grown in medium plus 1% of AOA Lusi. The composition of the medium AOA included KH_2PO_4, NH_4NO_3, $MgSO_4.7H_2O$, $FeCl_3$, $CaCl_2.2H_2O$, and mud. Streak plate method was used to purify bacteria.

The found colonies of bacteria then were observed and identified in morphology, gram staining and physiological tests (using Micro bacteria Identification System). Observation of macroscopic colonies of bacteria isolation included the colony shape, colony's color, and elevation. Gram staining is a multilevel painting that uses more than two kinds of substances and is done in stages to determine the gram shape and type of bacteria. For physiological test, the format was in the form of test-strip or microplate simple, of which was clearly visible as a result of reactions of different colours that can be interpreted using Microbact. Identification of organisms based on changes in pH and substrate usage. The data were analysed descriptively.

Phosphate solubilizing microorganisms are screened by a plate assay method using Pikovskaya (PVK) agar [9]. The qualitative analysis of phosphate solubilisation potential of selected Phosphate Soluble Bacteria (PSB) isolation was measured in vitro by determining available soluble phosphate in the Pikovskaya's broth supplemented with 0.5% TCP [10].

3. Results and Discussion

3.1. Physical and chemical properties of Lusi

Analysis of Total Petroleum Hydrocarbone (TPH) and nutrient levels of N, P, K, C/N ratio of Lusi are shown in Table 1.

Table 1. Chemical and physical properties of Lusi.

Parameter	Level	Category
TPH	41.000 mg/kg	Very high
N	0.20%	low < 0.21
P	0.01%	Very low < 10
K	0.22%	Low (0.1-0.2)
C	8.53%	Very high > 4
C/N ratio	42.7	Very high (>15)
Temperature	34 °C	
Humidity	60%	
pH	6.0	

The levels of TPH and C category are very high, therefore the value of the C/N ratio is also high (Table 1). It can be revealed that Lusi is contaminated by oil because TPH is a commonly used gross parameter for quantifying environmental

contamination originated by various petroleum hydrocarbon (PHC) products such as fuels, oils, lubricants, waxes, and others [11]. TPH is a complex compound consisting of alkane, aromatics, nitrogen, sulfur and asphaltenes fractions [12, 13]. While the levels of nutrients (N, P, and K) are in low or very low category. The data also portray that the temperature of location is hot proven by the pH score showing an acid category and a middle humidity level (Table 1). These parameters show that the nutrient status of Lusi is not appropriate to be used as a media for growing plants. Therefore, bioremediation using collaboration between multisymbiotic microorganism and plant can be conducted as well as the remediation of the structure and texture of the Lusi.

3.2. Isolation and identification of bacteria from Lusi

Based on the bacteria isolation in Lapindo mud, there are three bacteria isolations, which are further, characterized the colony. The characterization regards to the shape, colour, edge, elevation and optical (Table 2). In addition, this characterization is to know the difference among three colonies' characteristics. The isolated bacteria B101 and E104 have round shape, whereas the B106 one is into irregular shape.

Table 2. Characteristics of bacterial colony from Lusi.

Isolate	Shape	Colour	Edge	Elevation	Optical
B101	Round	Cream	Even	Convex	Opaque
B106	Irregular	Brown	Uneven	Slightly Convex	Opaque
E104	Round	Brown	Even	Convex	Opaque

3.3. Test of bacterial ability to solubilize phosphate and to degrade hydrocarbon

The three isolated bacteria obtained from the Lusi were then tested to get information regarding their ability in solubilizing phosphate. Tests were performed using Pikovskaya agar medium with composition of yeast extract, dextrose, calcium phosphate, ammonium sulphate, potassium chloride, magnesium sulphate, manganese sulphate, ferrous sulphate, and agar. The tests are done by growing bacterial isolation on Pikovskaya agar medium to isolate bacteria that can form clear zones around the colonies, of which further show the isolation capability of dissolving the available phosphate in the medium. Phosphate solubilizing bacteria are capable to produce organic acids that can dissolve minerals containing phosphate on phosphate. The results show that all the three (B101, B106, E104) of isolates tested showed positive results that can used phosphate.

These three bacteria are also tested to determine their ability to degrade the hydrocarbons. They were grown in medium supplemented with toluene AOA to determine its ability to grow on media containing hydrocarbons. After growing in three days after plating the bacteria on the medium, they are apparently still able to grow with a density of 10^5. It revealed that all of the three bacteria have also a potential to degrade and to grow in a hydrocarbon contaminated media (toluene). Toluene compound and some of its derivatives are toxic but microorganisms like *Pseudomonas, Bacillus,* and Actinomyces were able to survive in high toluene

concentrations and used toluene as a carbon source. Toluene degrading bacteria use different pathways for toluene consumption [14]. The oxidative microbes degrade toluene via hydroxylation of the aromatic ring to a mixture of catechols and cresols [15]**.** Toluene monooxygenase, benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase and catechol-2, 3-dioxygenase are enzymes involved in the degradation of toluene and are organized in two different pathways [16]. The key enzyme involved in this pathway is toluene dioxygenase [14].

3.4. The characterization of isolate bacteria

Identification of bacteria based on biochemical activity test is conducted by comparing the biochemical activity of each bacterium. Different bacteria have specific biochemical activity because each bacterium has a different enzymatic activity. Table 3 shows the data of biochemical/physiological properties bacteria found in Lusi. Physiological characteristics include the properties and the ability of bacteria to grow on media (Lysine, Ornithine, ONPG, indole, TDA, Gelatin, Arginine) H_2S, citrate, Urease, VP, (Glucose, Mannitol, Xylose, ONPG, Inositol, Sorbitol, Rhamnose, Sucrose, Lactose, Arabinose, Adonitol, Raffinose, Salicin - carbohydrates), oksidase, nitrate, motility. All isolates of B101, B106 and E104 have variety of results from the biochemical/physiological test (Table 3). Nevertheless, the three of isolate have ability to solubilize phosphate and have a potential to degrade the hydrocarbon.

Table 3. The biochemical and physiological test of isolate bacteria.

The Biochemical and Physiological	Isolate of Bacteria		
	B101	B106	E104
Oxidase	+	+	+
Mortility	+	-	+
Nitrate	+	+	+
Lysine	-	+	+
Ornithine	-	-	-
H_2S	-	-	-
Glucose	+	-	-
Mannitol	+	-	-
Xylose	+	-	-
ONPG	+	-	-
Indole	-	-	-
Urease	+	-	-
VP	+	-	-
Citrate	+	-	-
TDA	-	-	-
Gelatin	-	-	-
Malonate	+	-	-
Inositol	+	-	-
Sorbitol	+	-	-
Rhamnose	+	-	-
Sucrose	+	-	-
Lactose	+		-
Arabhinose	+	-	-
Adonitol	+	-	-
Raffinose	+	-	-
Salicin	+	-	-
Arginine	+	+	+

Table 4 showed that based on the gram staining, isolates B101, B106, and E104 are gram-negative bacteria. That is supported by previous finding that most of isolate phosphate solubilizing bacteria were gram-negative bacteria [17]. To distinguish the group of gram-positive or gram-negative bacteria based on the staining result of four reagents used in staining. On the basis of crystal violet colour application, all the cells will form the CV-I complex (Crystal Violet-Iodine) which will bind with Mg-RNA cell wall components, forming Mg-RNA complex-CV-I, which is not soluble in alcohol. Lugol solution amplifier as Mg-RNA complex-CV-I. Alcohol 95% as fat dissolving compounds will decolorize. In Gram (+) fat content a little, at the time of washing with alcohol, fat will dissolve and form a small pore proteins are then covered by dehydrated alcohol, so that the pore is closed. Consequently, tough primary dye washed and the cells become purplish blue. Grams of fat in cells (-) a lot, so the time of dissolution of alcohol produces large pores that cannot be covered by a porous dehydrated, consequently alcohol wash all Mg-RNA complex-CV-I and cell loss of colour. Reagent, which is fourth in Safranin staining, were used to replace the basic colours that have been lost due to alcohol leached, Gram (-) will be coloured red and Gram (+) remained purple.

Table 4. Species of bacterial isolate from Lusi.

Code of Bacterial Isolation	**Code Microbact 2000**	**Identification Result**	
		Species Name	Percentage of Probability
B101 (gram negative)	707563777	*Pseudomonas pseudomallei*	99.99%
B 106 (gram negative)	540000101	*Pseudomonas fluorescens-35*	58.26%
E104 (gram negative)	740002001	*Pseudomonas stutzeri*	66.40%

The species of bacteria were isolated and then identified from the Lusi, are three species namely *Pseudomonas pseudomallei, Pseudomonas fluorescens-35,* and *Pseudomonas stutzeri*. It indicates that hydrocarbon biodegradation by a microbial community depends on community composition and adaptive response to the presence of hydrocarbons [18, 4]. It means that when these indigenous bacteria are used in bioremediation process, the bacterial adaptation is a key role to determine the existing bacteria in environmental conditions as well as how these bacteria have mechanisms to limit the unfavourable conditions for the survival of the bacteria. Only bacteria, that were able to increase its role in improving oil contamination can be fit their role. This indicates that the success of microbes to degrade hydrocarbons is highly dependent on its ability to break down the carbon [19]. Thus, it can be said that the three species of bacteria that can be isolated from the Lusi is a species of indigenous bacteria that have great adaptive power of the soil conditions in the mudflow.

Besides Mycorrhizae, phosphate soluble bacteria is able to increase the availability of Phosphate in the environment by dissolving insoluble P (the organic acids) to be absorbed by plants. Some types of bacteria and fungi that have this capability include *Pseudomonas, Mycobacterium, Micrococcus, Flavobacterium, Penicillium, Sclerotium,* and *Bacillus*. These microbial produce organic acids such as citric acid, glutamate, succinate, lactate, oxalate, gliocsalat, malic, fumaric, tartaric, and α ketobutirat [20]. Increased organic acids are

normally followed by a decrease in pH, resulting in the dissolution of Ca-bound P. Decreasing in pH can also be caused by release of nitric and sulfuric acid in the oxidation of sulfur and ammonium chemoautotrophic, by Thiobacillus bacteria and Nitrosomonas respectively.

Meanwhile, other researchers proved that some microbes are able to use carbon as a source in the process of hydrocarbon compounds including *Pseudomonas, Bacillus, Acinetobacter, Alcaligenes, Xanthomonas, Benecdea, Brevi bacterium, Methvlobacterium, Methylococcus, and Mycobacterium* [21]. They have isolated oil-degrading bacteria in Dumai-Riau Province, Indonesia, such as *Pseudomonas sp, Flavobacterium sp,* and *Micrococcus sp*. Based on the previous studies, the isolate bacteria identified have the ability to dissolve phosphate. However, because these bacteria isolated from oil contaminated soil, then it is at the same time indicates that these bacteria also had the ability to degrade hydrocarbons contained relatively high amounts of oil contaminated soil, were used as indicator of TPH and C in a high level (Table 1). This is understandable that the bacteria will meet the energy that needs to grow and carry out metabolic activities by using the resources available in their environment. For all three of these bacteria use a relatively high hydrocarbon compounds in their environment.

Generally, the phosphate solubilizing microorganisms (PSM) play a very important role in phosphorus nutrition by exchanging its availability to plants through release from inorganic and organic soil phosphorus pools by solubilisation and mineralization. The main mechanism in the soil for mineral phosphate solubilisation is by lowering the soil pH by the microbial production of organic acids and mineralization of organic phosphorus by acid phosphates [6, 22-24].

It is also known that the bacteria were found in Lusi also to have the ability to degrade hydrocarbons, it must be understood that petroleum biodegradation is actually a natural process, which involves microorganisms that can transform the petroleum hydrocarbons into simpler components. The mechanism of transformation and decomposition by microorganisms carried one example is the n-alkane hydrocarbon solution. By solving n-alkane hydrocarbon molecules by microorganisms is initiated by complex multi mono-oxygenized enzyme system (ω-hydroxylase) that can oxidize alkanes to alcohols primary. Furthermore, primary alcohols are oxidized to form aldehyde compound and eventually into fatty acids. The resulting fatty acids can be directly decomposed into CO_2 through an oxidation process or used as a nutrient (carbon and energy source) for cell growth via β-oxidation process. CO_2 is formed, some will react with petroleum fractions and petroleum led to be inflated, so that the surface tension and viscosity will decrease [4].

Bacteria require carbon molecules as a source of nutrients and energy for metabolism and breeding. In particular, a group of microorganisms that are able to use carbon sources derived from hydrocarbon compounds called hydrocarbonoclastic microorganisms [25]. The genus of bacteria that is the most important hydrocarbon users based on the frequency of isolation was *Achromobacter, Acinetobacter, Aeromonas, Corynebacterium, Flavobacterium, Methylobacter, Methylobacterium, Pseudomonas* [4]. Characteristics hydrocarbonoclastic microorganisms that are not owned by other microorganisms is the ability to express ω-hydroxylase enzyme, the enzyme oxidizing hydrocarbons, so that the bacteria are able to degrade petroleum hydrocarbons by deducting the hydrocarbon chain becomes shorter [26].

There are several investigations showing the beneficial effects of hydrocarbonoclastics species of the genus *Pseudomonas* in plants growth, through hydrocarbons degradation and toxic effects reduction. For example, some researchers found that *Pseudomonas fluorescens* BBN1 and *Rhodococcus qingshengii* BBG1 together, reached degradation rates of 95% (n-dodecane), 66% (toluene) and 70% (naphthalene) of the contaminants initial concentration at 42 days [27]. Other example, some researchers found that found an increase in roots dry weight of *Lepironia mucronata*, cultivated in a petroleum-contaminated soil and inoculated with the hydrocarbonoclastics bacteria *Alcaligenes faecalis* and *Pseudomonas alcaligenes* [28].

4. Conclusion

Some concluding observations from the investigation on bacterial isolation and identification in the Lusi are given below.

- The levels of TPH and C category are very high, while the levels of nutrients (N, P, and K) are in low or very low category.
- There are three bacteria isolations which are further characterized the colony to know the difference among three colonies' characteristics. The isolated bacteria B101 and E104 have round shape, whereas the B106 one is into irregular shape. All three isolates are gram-negative bacteria.
- Bacterial isolation and identification in the Lusi were *Pseudomonas pseudomallei, Pseudomonas fluorescens-35*, and *Pseudomonas stutzeri*. These three bacterial isolations have the ability to solubilize phosphate and simultaneously have a potential to degrade hydrocarbon compound.

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