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Bioremediation model of oil-contaminated soil in Lapindo mud using multisymbiotic organism

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Abstract

Purpose – The study aimed at developing the bioremediation model of Lapindo mud through multisymbiotic organism.

Design/methodology/approach – The research was conducted using completely randomized design. The model plants chosen in this research were soybean. The interaction pattern during the treatment was used to develop the bioremediation model based on the parameters.

Findings – The results showed that there was an effect of the type of organism on the parameters, namely: the growth of plant (biomass, height, length of root, and number of leaves), the biomass of root nodules, the percentage of mycorrhizal infection, the content of water, nitrogen, phosphorus, and total petroleum hydrocarbons (TPHs). There was a pattern of multisymbiotic interaction between each organism and roles of each symbiont in that interaction. Therefore, the plants were capable of surviving in the environment of Sidoarjo Lapindo mud. This pattern can be named as the bioremediation model proposed, which is the analogy of tripartite symbiosis between plants, mycorrhizae, and *Rhizobium* but also adding plant growth bacteria such as phosphate-solubilizing bacteria and hydrocarbon degradation bacteria. The implementation of this model can be used to treat oil-contaminated soil in order to be used as a plant growth medium.

Originality/value – Phytoremediation is a new and promising approach to remove contaminants in the environment but using plants alone for remediation confronts many limitations. Therefore, the application of plant-growth-promoting rhizobia (PGPR) has been extended to remediate contaminated soils in association with plants (Zhuang *et al.*, 2007). The development of the model will use the analogy of tripartite symbiosis between plants, mycorrhizae, and *Rhizobium*. The developed model will be based on the interaction pattern on each parameters obtained. Bioremediation is chosen because it is considered an effective technique to transform toxic components into less toxic products without disrupting the surrounding environment. Besides, bioremediation is cheaper and environment-friendly because it utilizes microorganisms to clean pollutants from the environment (Nugroho, 2006).

Keywords Bioremediation model, Sidoarjo Lapindo mud

Paper type Research paper

1. Introduction

A mud volcano disaster in 2005, due to oil and gas drilling, made some regions at Lapindo Sidoarjo, East Java, Indonesia, sink and the surrounding area cannot be used even if it has potential for agricultural areas. Since the beginning of the event until October 2008, it was predicted that the flowing rate of the mud was ranging from 100,000 to 180,000 m^3 per day (Plumlee *et al.*, 2008; Jalil *et al.*, 2010; Manzzini *et al.*, 2012) The environment is exposed to various compounds of petroleum, which is a potential major pollutant in the environment. This pollution is also caused by many potential harmful substances in the mud such as total petroleum hydrocarbons (TPHs), heavy metals, and polycyclic aromatic hydrocarbons (PAHs). PAHs are the primary inorganic contaminant, which includes lead, cadmium, and chromium in an exceed threshold. These conditions will be a negative influence for plant growth and development (Rojo-Nieto and Perales-Vargas-Machuca, 2012; Nie *et al.*, 2011; Peng *et al.*, 2009). Therefore, the efforts should be conducted to exclude these negative effects.

Plant–bacteria symbiosis has been extensively studied and applied to improve crop yield. This symbiosis is effective to remediate the hydrocarbon-polluted soil. It is well known that the degradation of hydrocarbon pollutants depends mainly on the presence and metabolic activities of plant-associated rhizome- and endophytic bacteria possessing specific genes required (Khan *et al.*, 2013).

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The removal of organic pollutants was increased by the addition of plant-growth-promoting rhizobia (PGPR), probably, by enhancing plant germination and survival in heavily contaminated soils and by stimulating the plant to grow faster (Huang *et al.*, 2004a, b). Thus, the combination of specific contaminant-degrading bacteria and PGPR was effective to solve a contaminant problem in the soil such as TPHs. Nevertheless, there were very few studies concerning the application of PGPR for the environmental remediation with plant (Lucy *et al.*, 2004).

PGPR can stimulate plant yields because its ability to convert insoluble phosphate compounds that are available to be absorbed by plant (Igual *et al.*, 2001; Rodriguez *et al.*, 2006). The concentration of soluble P in soil is usually very low (Mikanova and Novakova, 2001). It is well known that *Pseudomonas, Bacillus, Rhizobium, Micrococcus, Aerobacter, Flavobacterium, Achromobacter, Erwinia* have the ability to solubilize insoluble inorganic phosphate compound (Rodriguez *et al.*, 2006). Organic substrates in the soil can be a source of *P* for plant growth by hydrolyzing to inorganic *P*. Mineralization of the most organic phosphorous compound is carried out by enzymes such as *phytase* (Richardson and Habodas, 1997), *phosphatase (phosphohydrolase)* (Rodriguez and Fraga, 1999).

On the other side, arbuscular mycorrhizal fungi (AMF) are rhizosphere microorganisms that form mutual symbiosis with the root system (Smith and Read, 1997). This symbiosis has a role on the phytoremediation of soils contaminated with inorganic and organic compounds (Joner and Leyval, 2003a, b; Meharg, 2001). The role is related to enhanced plant adaptation and growth, including abiotic and biotic stress resistance and enhanced nutrition. In addition, the AMF may include modification of microbial groups in the mycorrhizosphere and the potential proliferation of petroleum-degrading microorganisms via extraradical hyphal exudation (Gaspar *et al.*, 2002). The results of recent studies revealed that the symbiosis of plant roots with AMF may also be efficient for the phytodegradation of organic-compound-contaminated soil (Gao *et al.*, 2010, 2011; Hassan *et al.*, 2014; Rajtor and Piotrowska-Seget, 2016).

Phytoremediation is a new and promising approach to remove contaminants in the environment but using plants alone for remediation confronts many limitations. Therefore, the application of PGPR has been extended to remediate contaminated soils in association with plants (Zhuang *et al.*, 2007). The result of bioremediation in the petroleum-contaminated soil at Bojonegoro, East Java, Indonesia, using soybean as a model plant grown on these soils showed that there is a positive effect of combination of hydrocarbon-degrading bacteria, phosphate-dissolving bacteria, *Rhizobium*, and mycorrhizae in declining the hydrocarbon, increasing the ratio of nitrogen and phosphorus, the percentage of mycorrhizal infection and root nodules, and also increasing the growth of plants in that land (Rahayu *et al.*, 2010). This finding reveals that the utilization of multisymbiotic organism, which involves on interaction between phosphate-solubilizing bacteria (PSB), hydrocarbon-degrading bacteria, mycorrhizae, and nitrogen fixation bacteria, and also soybean as a model plant, plays an important role, including reservation of structure and texture of Lapindo mud before it is utilized as a medium of growth. Phytoremediation of organic pollutants depends on plant–microbe interactions in the rhizosphere of the plants, but the extent and intensity of such rhizosphere effects are likely to decrease with increasing distance from the root surface (Das and Chandran, 2011). A utilization of plants in bioremediation of oil-contaminated soil with high PAHs is rarely conducted, whereas a biodegradation of contaminant compounds in the soil can be increased by an effective vegetation in the depth of 0–20 cm (Keller *et al.*, 2008) in which the quality of Rhizodeposition is also dependent on the vegetation (Jones *et al.*, 2004). On other hand, plants should be native to the oil-contaminated area, and they should be tolerant to the conditions of weather and soil properties (Reynoso *et al.*, 2008). Lebrazi and Fikri (2018) found that the use of bacterial genetic/molecular engineering approaches, particularly for the Rhizobium–legume symbiotic association, has proved to be an interesting

and significant alternative. It offers a greater degradation capacity of various metal contaminants to promote contaminated soil remediation. Therefore, the role of multisymbiotic soil microorganism with soybean was used to develop the bioremediation model of Lapindo mud. The development of the model will use the analogy of tripartite symbiosis between plants, mycorrhizae, and *Rhizobium*. The study will describe how the bioremediation model will be developed and fit to overcome the area di Lapindo, Sidoarjo, East Java, Indonesia, which has the crude oil pollutant as a majority. The developed model will be based on the interaction pattern of each parameters obtained.

Bioremediation model was chosen in order to analyze the pattern in the multisymbiotic relationship between organisms and the role of each symbiont in the relationship pattern that made plants survive in environmental condition such in Lapindo Sidoarjo Mudflow. The proposed bioremediation model can be used to determine biological agents that can improve Lapindo mud in terms of physics, chemistry, soil biology, to make it feasible to be utilized as a planting medium for cultivated plants.

2. Methods

In the first stage, an experiment was conducted to find the Lapindo mud bioremediation model through the use of multisymbiotic organisms by combining effective organisms: mycorrhiza – hydrocarbon-degrading bacteria – phosphate – rhizobium solvent bacteria tested on legume plants. These multisymbiotic organisms are indigenous organisms that have been found in previous studies. In the second stage, an experiment was conducted for a field trial in the Lapindo mud area on a bioremediation model that had been prepared at once. Experiment method was conducted to develop the bioremediation model of Lapindo mud at Sidoarjo, East Java, Indonesia, through a utilization of multisymbiotic organisms by combining effective organisms, namely mycorrhizae, hydrocarbon-degrading bacteria, *Rhizobium,* and phosphate-dissolving bacteria, which are tested in the legumes. Those multisymbiotic organisms are indigenous organisms found in the previous research. The bacteria isolate used in this study were *Pseudomonas pseudomallei, Pseudomonas fluorescens, Pseudomonas stutzeri*, and *Rhizobium japonicum*. Meanwhile, mycorrhizae used are *Glomus mosseae*.

Completely randomized design was used to analyze a pattern of organism combination. The factor used in this study was a combination of organisms that will be tested in the legumes with three times replication. Data obtained were the TPHs in the soil, the concentration of phosphorus and nitrogen, the percentage of mycorrhizal infection, the number of effective root nodules, and the growth of legumes (height, biomass, the length of root, and a number of leaves).

The subjects of this research are Lapindo mud, legume plants, phosphate-solubilizing bacteria, hydrocarbon-degradation bacteria, Rhizobium, and arbuscular vesicular mycorrhiza. The data were analyzed using one-way ANOVA and the least significant difference (LSD) test and qualitative descriptive analysis.

3. Results

3.1 Soybean growth

Table I shows the parameters of soybean growth, which consist of its height, biomass, the length of root, and the number of leaves.

3.1.1 Biomass of soybean. The data shows that the treatment of several organism combinations influenced the biomass of soybean. There was a significant difference between MB3, MR, RB3, MB1 with B2B3 and RB1B2 (Table I). Meanwhile, the other treatments are not significantly different. It is also known that the treatment of RB1B2 gave the highest biomass;

No	Treatment	Growth of soybean			
		Height (cm)	Biomass (g)	Length (cm)	Leaves number
1	M	47.83 abcde	1.10 ab	15.33 a	9.00 abcde
2	R	21.00 ab	0.37 ab	7.17 a	2.67 a
3	B1	49.75 abcd	4.15 abc	52.00 b	19.50 abcde
4	B2	23,33 ab	1,30 ab	14,30 a	8.00 abcde
5	B3	45,33 abcd	2,33 ab	19,70 a	12.67 abcde
6	MR	16,67 a	0,20 a	8,20 a	6.67 abcde
7	MB1	34.67 abc	0.23 a	6.00 a	6.00 abcd
8	MB2	55.67 abcde	2.93 abc	12.00 a	16.00 abcde
9	MB3	18.50 a	0.15 a	5.67 a	4.33 ab
10	MRB1B3	64.00 bcde	3.50 abc	12.67 a	19.00 abcde
11	MRB2B3	46.40 abcd	1.37 ab	7.17 a	10.67 abcde
12	MB1B2	107.00 fg	3.34 abc	9.33 a	20.33 bcdef
13	MB1B3	108.00 fg	2.97 abc	16.67 a	21.00 cdef
14	MB2B3	73.33 cdef	3.47 abc	6.17 a	17.00 abcde
15	RB1B2	123.33 g	7.50 d	16.00 a	35.00 f
16	RB1B3	49.33 abcde	2.90 abc	8.40 a	13.67 abcde
17	RB2B3	50.67 abcde	1.00 ab	17.00 a	9.33 abcde
18	RB1	64.00 bcde	2.43 abc	12.00 a	15.00 abcde
19	RB2	32.67 abc	1.70 abc	8.40 a	11.00 abcde
20	RB3	33.33 abc	0.23 a	5.00 a	8.00 abcde
21	B1B2	33.67 abc	0.83 ab	6.67 a	6.67 abcde
22	B1B3	49.00 abcde	1.83 bc	8.67 a	10.67 abcde
23	B2B3	56.00 abcde	4.73 c	15.67 a	23.00 ef
24	MRB1	36.00 abcd	0.43 ab	9.00 a	10.33 abcde
25	MRB2	41.77 abcd	0.90 a	9.17 a	4.67 abc
26	MRB3	90.67 efg	3.70 bc	12.00 a	21.67 def
27	MRB1B2	79.67 def	2.90 abc	11.17 a	15.33 abcde
28	Control	26.33 ab	0.70 ab	9.67 a	7.00 abcde

Note(s): M: Mycorrhizae *(Glomus mosseae)*; R: *Rhizobium japonicum*; B1: *Pseudomonas pseudomallei*; B2: *Pseudomonas flourescens*; B3: *Pseudomonas stutzeri*; Data of soybean growth in 90 days after planting

Table I. Result of Duncan test on the parameters of soybean growth

meanwhile, the treatments of MB3 and MR gave the lowest biomass. Data indicates that compared to only one organism or a combination of two organisms, the combination of three organisms not only between mycorrhizae and bacteria (MB1B2, MB1B3) but also between *Rhizobium* and bacteria (RB1B2) or between *Rhizobium* and bacteria (*M*, *R*, B3) gave the best biomass of soybean.

3.1.2 Height of soybean. The organism combination also affected significantly the height of soybean. There was significant difference between MR, MB3, and MB1B2, RB1, MRB3, RB1B2, and MRB1B2. Meanwhile, the other treatments are not significantly different. It is also revealed that the treatment of RB1B2 gave the highest height of soybean; meanwhile, the treatment of MR gave the lowest height. Data indicates that compared to only one organism (*B*, *M*, *R*) or combination of two organisms, the combination of three organisms not only between mycorrhizae and bacteria (MB1B2, MB1B3) but also between *Rhizobium* and bacteria (RB1B2) or between *Rhizobium* and bacteria (*M*, *R*, B3) gave the best height of soybean.

3.1.3 Length of soybean root. The result reveals that the treatment of organism combination also affected significantly the length of soybean root. There was significant difference between B1 and all treatments; meanwhile the other treatments are not significantly different. According to Table I, it is also known that the treatment of B1 gave the highest length of soybean root; meanwhile, the treatment of RB3 gave the lowest length of soybean root.

3.1.4 Number of soybean leaves. The result shows that the treatment of organism combination affected the number of soybean leaves. Then, a further analysis using Duncan test was conducted to know the difference of each treatment showing that there was difference between B2B3 and RB1B2; meanwhile the other treatments are not significantly different. Table I also shows that the treatment of MRB3 gave the biggest number of soybean leaves; meanwhile, the treatment of *R* gave the smallest number of soybean leaves. Data indicates that compared to only one organism (*B*, *M*, and *R*) or a combination of two organisms, the combination of mycorrhizae and bacteria (MB1B3) gave the best number of soybean leaves.

3.2 Root nodules and percentage of mycorrhizal infection in soybean

Treatment of MRB1B3 gave the highest biomass, while the treatment of MB1 gave the lowest biomass (Table II). Furthermore, treatments of *R*, RB1B2, and MRB1B3 produced the highest root nodules. The other treatments failed to produce active root nodules. Then, the treatments of MB1B3 and MRB3 gave the highest percentage of mycorrhizal infection.

3.3 Water content, nitrogen, phosphorus, and TPH concentration

3.3.1 Water content. Table III indicates that the water content in soybean was significant, which means that the organism combination also affected the water content in soybean.

No	Treatment	∑ Biomass of root nodules (g)	Biomass of active nodules (g)	Biomass of inactive nodules (g)	Percentage of mycorrhizal infection (%)
1	M	–	–	–	41.43
2	R	0.20	0.17	0.03	–
3	B1	–	–	–	–
4	B2	–	–	–	–
5	B3	–	–	–	–
6	MR	–	–	–	–
7	MB1	0.09	0	0.09	–
8	MB2	0.14	0.06	0.08	–
9	MB3	–	–	–	–
10	MRB1B3	0.20	0.12	0.08	31.97
11	MRB2B3	0.12	0.06	0.06	44.78
12	MB1B2	–	–	–	32.35
13	MB1B3	0.14	0.04	0.10	48.62
14	MB2B3	0.10	0	0.10	21.57
15	RB1B2	0.17	0.15	0.02	–
16	RB1B3	–	–	–	–
17	RB2B3	0.09	0.07	0.02	–
18	RB1	–	–	–	–
19	RB2	–	–	–	–
20	RB3	–	–	–	–
21	B1B2	0.09	0.02	0.06	–
22	B1B3	0.09	0.02	0.06	–
23	B2B3	–	–	–	–
24	MRB1	–	–	–	–
25	MRB2	–	–		32.26
26	MRB3	0.12	0.08	0.05	51.72
27	MRB1B2	0.15	0.10	0.05	–
28	Control	–	–	–	–

Table II.
Root nodules and percentage of mycorrhizae infection in soybean

Note(s): *M*: Mycorrhizae (*Glomus mosseae)*; *R*: *Rhizobium japonicum*; B1: *Pseudomonas pseudomallei*; B2: *Pseudomonas fluorescens*; B3: *Pseudomonas stutzeri*

No	Treatment	Water content (%)	Nitrogen concentration (%)	Phosphorus concentration (%)	TPH concentration (mg/Kg)
1	M	15.61 abcdef	0.18 a	3.64 c	30.00 gh
2	R	21.82 defghi	1.03 bcdef	0.64 ab	29.00 fgh
3	B1	17.33 bcdef	0.60 abcd	0.98 ab	36.50 hi
4	B2	8.75 a	0.60 abcd	0.84 ab	21.00 efg
5	B3	15.55 abcdef	0.66 abcde	0.32 a	9.00 abcd
6	MR	18.72 bcdefg	0.84 abcdef	0.84 ab	15.00 bcde
7	MB1	16.57 abcdef	1.30 cdef	0.39 ab	18.00 cde
8	MB2	16.42 abcdef	1.04 bcdef	0.72 ab	11.00 abcde
9	MB3	14.40 abcde	0.74 abcde	0.88 ab	8.00 abc
10	RB1	16.99 bcdef	0.45 ab	0.49 ab	12.00 i
11	RB2	13.74 abcd	0.36 ab	0.31 a	5.00 cde
12	RB3	18.48 bcdefg	0.68 abcde	0.33 a	3.00 bcde
13	B1B2	16.25 abcdef	0.58 abc	0.29 a	18.00 ef
14	B1B3	11.37 ab	0.66 abcde	0.29 a	19.00 cde
15	B2B3	19.92 cdefgh	0.39 ab	0.33 a	17.00 abcde
16	MRB1	12.84 abc	0.91 abcdef	0.24 a	18.00 fgh
17	MRB2	21.87 defghi	0.49 ab	0.31 a	14.00 efg
18	MRB3	19.07 bcdefgh	0.91 abcdef	0.30 a	42.00 abcde
19	MRB1B2	22.50 efghi	0.90 abcdef	0.42 ab	18.00 abcd
20	MRB1B3	27.77 i	1.14 bcdef	0.88 ab	15.00 abcde
21	MRB2B3	26.01 ghi	0.74 abcde	0.35 ab	20.00 ab
22	MBIB2	23.16 fghi	0.97 bcdef	2.01 abc	17.00 a
23	MB1B3	26.89 hi	1.10 bcdef	2.25 bc	11.00 cde
24	MB2B3	16.15 abcdef	1.59 f	0.73 ab	29.00 de
25	RB1B2	17.57 bcdef	1.38 def	1.74 ab	21.00 cde
26	RB1B3	17.57 bcdef	1.39 ef	0.48 ab	11.00 cde
27	RB2B3	14.30 abcd	1.37 def	0.40 ab	9.00bcde
28	Control	20.33 cdefghi	0.94 abdef	3.64 c	11.00 abcde

Note(s): *M*: Mycorrhizae (Glomus mosseae); *R*: Rhizobium japonicum; B1: Pseudomonas pseudomallei; B2: *Pseudomonas fluorescens*; B3: *Pseudomonas stutzeri*

Table III.
Water content, nitrogen, phosphorus, and TPH concentration

It also shows that there was significant difference between MRB2/B2B3 and RB3 while the other treatments are not significantly different. The highest water content in soybean was found in the treatment of B2B3; meanwhile, the lowest water content in soybean was obtained in the treatment of B2.

3.3.2 Nitrogen concentration (N). Table III indicates that the organism combination also influenced significantly the concentration of nitrogen in soybean. Then, there was significant difference between M with MRB3 and MRB1 while the other treatments are not significantly different. The highest concentration of nitrogen in soybean was in the treatment of MRB3; meanwhile, the lowest concentration of nitrogen in soybean was obtained from the treatment of *M*.

3.3.3 Phosphorus concentration (P). The organism combination affected significantly the phosphorus concentration in soybean. There was treatment difference between RB1B3/B2B3 and *M* while the other treatments are not significantly different. The highest concentration of phosphorus in soybean was in the treatment of *M*; meanwhile, the lowest concentration of phosphorus in soybean was obtained from the treatment of RB1B3.

3.3.4 TPH concentration. The concentration of TPH in the medium of growth was also analyzed to elucidate the effectivity of organism variation to decrease the TPH in the medium of growth. Table III shows the analysis result of the concentration of TPH in the medium of growth. The result of Duncan test indicated that there was treatment difference between

MB1B2/B1 and RB1 while the other treatments are not significantly different. According to Table III, it can be known that the highest concentration of TPH was in the treatment of MRB3; meanwhile, the lowest concentration of TPH was obtained from the treatment of RB3.

4. Discussion

It shows that the concentration of TPH at the Lapindo mud is 41 mg/kg; therefore, it can be stated that Lapindo mud is contaminated by oil. It has been already known that legumes are unique plants because they can fix the free nitrogen by using the root nodules bacteria. In the symbiosis between legumes with bacteria of root nodules and mycorrhizae (tripartite symbiosis), every symbiont has a different role. Plants provide nutrients for *Rhizobium* and mycorrhizae; *Rhizobium* provides nitrogen for plants through nitrogen fixation and mycorrhizae shares phosphorus for plants and *Rhizobium*. This pattern gives information of mechanism pattern of symbiosis in legumes, so the plants are capable of surviving in the environment.

The analogy of this symbiosis pattern was used to explore the pattern between hydrocarbon-degrading bacteria, PSB, *Rhizobium*, and mycorrhizae in soybean, expected to be able to use to develop the bioremediation model of Lapindo mud by using benefits obtained from the interaction of multisymbiosis between all organisms and the roles of each symbiont in that interaction, so the plants can survive from the oil-contaminated soil.

4.1 Growth of plants

Data indicates that compared to combination of two organisms or only one organism, generally the combination of three organisms, mycorrhizae and bacteria, *Rhizobium* and bacteria, or mycorrhizae, *Rhizobium*, and bacteria gave the best effect on the biomass, the height of plants, the length of root, and the number of leaves in soybean (Table I). The rhizospheric microorganisms like AMF are able to contribute to improve plant adaptation, growth, and nutrition under environmentally adverse condition such as petroleum-contaminated soil (Davies *et al.*, 2001; Amaya-Carpoi *et al.*, 2005; Franco-Ramirez *et al.*, 2007; Nadeem *et al.*, 2014; Bowles *et al.*, 2016). Many studies reported that in the hydrocarbon-contaminated soils, AMF can improve plant biomass (Trejo *et al.*, 2013; Nwoko, 2014; Lu and Lu, 2015), shoot and root length (Aranda *et al.*, 2013), *N* and *P* uptake (Hernandez-Ortega *et al.*, 2012; Zhou *et al.*, 2013).

Recently, some ecologists have found that phytoremediation with the aid of mycorrhizae can enhance efficiency in the removal of toxic metals; therefore, AMF plays an important role for developing phytoremediation program in metal-contaminated soil. AMF can facilitate the survival of plants growing on metal-contaminated soil by enhancing their nutrient acquisition, protecting them from the metal toxicity, absorbing metals, and also enhancing phytostabilization and phytoextraction, protecting them from the metal toxicity, absorbing metals, and also enhancing phytostabilization and phytoextraction (Leung *et al.*, 2013).

Microbes, which are capable of decomposing hydrocarbon compound of oil and solubilizing phosphate, are utilized in this study. There were three isolates used, which were obtained from Lapindo mud. The hydrocarbon-degrading bacteria gave the possibility that the abundance of hydrocarbon in Lapindo mud can break down to become useful compounds for plants. In addition, these three isolates used in this study have also the ability in solubilizing the fixed form of phosphorus. The release of insoluble and fixed phosphorus plays an important role not only to reduce negative environmental impact but also to increase soil phosphorus availability and soil fertility. Insoluble phosphate can be solubilized in acidic soils by microbial activity (Bashan *et al.*, 2013). In fact, a group of soil bacteria called PSB have the ability to dissolve part of the fixed and insoluble phosphorus and make it available to the

crop by producing enzymes and low-molecular-weight organic acids (Khan *et al.*, 2009; Tilak *et al.*, 2005; Rodríguez and Fraga, 1999).

For growth and development, plants need phosphorus as an essential macronutrient for conducting metabolic pathways such as photosynthesis, biological oxidation, nutrient uptake, and cell division (Illmer and Scinner, 1992). Phosphate-solubilizing microorganisms play an important role not only in biogeochemical phosphorus cycling in terrestrial and aquatic environments (Das *et al.*, 2007) but also in balancing plant nutrition, because these strains convert the insoluble phosphate into soluble forms (Mishra *et al.*, 2016). Soil fertility can be increased by converting insoluble *P* to soluble *P* by releasing chelation, organic acids, and ion exchange (Whitelaw, 2009; Narula *et al.*, 2000). The positive effect of PSB has been found by increasing *P* soil availability, *P* uptake in plant, and plant growth (Gulati *et al.*, 2009; Guptaa *et al.*, 2012).

The availability of nitrogen, phosphorus, and zinc can stimulate the plant growth. Nitrogen is involved in the existence of amino acid and protein, which also affects the metabolism process controlled by enzymes. Phosphorus also has a role in the metabolism process, which involves energy in the form of ATP. Furthermore, both nitrogen and phosphorus are involved in the construction of plant cell structure such as cell membrane, a structural protein, and histone protein. Meanwhile, cuprum and zinc are actively involved in the electron transport of photosynthesis process. Therefore, it can be understood that the involvement of three organisms influenced positively the growth of plants.

4.2 Root nodules and mycorrhizal infection

The analysis result of a number of root nodules, the biomass of active nodules, the biomass of inactive nodules, and percentage of mycorrhizal infection (Table II) showed that the formation of root nodules in soybean was influenced by the existence of *Rhizobium* in the medium, the interaction with other bacteria, and the existence of mycorrhizae. Meanwhile, the percentage of mycorrhizal infection depended on the existence of mycorrhizae. The interaction with other bacteria also gave better results. This phenomenon also happened in the spores of mycorrhizae. However, the interaction of every organism showed that organisms in Lapindo mud can interact with the environment so they can survive in the condition of Lapindo mud.

The plants have an important role in the phytoremediation of organic pollutants concerning the extent and intensity of rhizosphere and plant–microbe interactions in the rhizosphere (Joner and Leyval, 2003b), especially when the plant is a native from oil-contaminated area (Reynoso *et al.*, 2008). Vetch and mustard fostered the removal of final TPHs concentration and petroleum hydrocarbon (PHCs) from soil, which could be 15.6 percent and 12 percent lower than that in the unplanted soil. The two crops elicited the greatest root degradation activities and sustained particularly great populations of rhizosphere bacteria that are known as hydrocarbon degraders (Smith *et al.*, 2005). The rhizoremediation of many grass species, such as mulberry, vetch and mustard, alfalfa, and rape has been found to remove the hydrocarbons (Smith *et al.*, 2005; Kamath *et al.*, 2005). It seems that the synergism of plant and soil microorganism interaction plays an important role in plant adaptation; thus, rhizoremediation can work effectively.

4.3 Concentration of water, nitrogen, phosphorus, and TPH in the medium of growth

The concentration of water, nitrogen, phosphorus, and TPH in the medium of soybean indicates in a variety value. The highest water content in the plant was found in the treatment of combination of two bacteria. The treatment of combination of three organisms (*M*, *R*, and B3) affected the highest nitrogen concentration. The biggest concentration of phosphorus was found in three treatments of mycorrhizae, PSB, and hydrocarbon-degrading bacteria.

Meanwhile, the smallest concentration of TPH was found in the treatment of *Rhizobium* and bacteria. Data indicates that every organism (mycorrhizae, *Rhizobium*, PSB, and hydrocarbon-degrading bacteria) has a different response when it interacts with other organisms (Table III).

The availability of nitrogen and phosphorus and the decrease of TPH concentration in the medium of growth (Lapindo mud) depend on the role of every organism, namely mycorrhizae, *Rhizobium*, and hydrocarbon-degrading bacteria, which is also able to solubilize phosphate. The decrease of TPH concentration depends on the degradation of hydrocarbon compounds by bacteria, which are added to the petroleum-contaminated soil. The decrease of TPH was caused by several factors, not only because of the bacteria's ability in decomposing hydrocarbon but also environmental factors that stimulate the degradation of hydrocarbon.

Three isolates of bacteria used in this study are active indigenous microorganisms in Lapindo mud, which have the capability of degradation of several pollutants, such as petroleum hydrocarbon. The bacteria decompose carbon organic materials and use them as the energy resources so the carbon organic materials in the petroleum-contaminated soil can be decreased. In addition, the bacteria is capable of decomposing aromatic hydrocarbon component, for example, benzene and toluene (Liu *et al.*, 2012; Huang, 2004a, b).

Mechanism of benzene biodegradation was initiated by the termination of aromatic rings by dioxygenase enzyme. Bacteria formed the dihydro diol compound in the aromatic component with a single ring. Then, bacteria metabolize and produce catechol or procaterol compounds. Those compounds are broken down in ortho pathway or meta-cleavage pathway. Ortho pathway cuts down the aromatic core of catechol or procaterol between two hydroxyl clusters becoming muconate and gluconolactone. Then, the metabolism of 4-oxiadipate enol-lactone occurs followed by 3-oxiadipate (β-cento adipate) and continued by Kreb cycle producing compound between acetyl-CoA and succinate. On another side, meta-cleavage pathway cuts down the ring next to two hydroxyl groups and produces 2-hydroxil-muconic semialdehyde. The products of metabolism are pyruvate acid, format acid, acetaldehyde acid, which enter the Kreb cycle and finally produce H_2O, CO_2, and continuing compounds (Das and Chandran *et al.*, 2011; Kumar *et al.*, 2011).

Besides benzene, degradation of phenol compound can be conducted easily than degradation of a derivate synthetic compound or aromatic homolog causing by decomposing bacteria so this bacteria is better than its degradation of the derivate synthetic compound. The termination of phenol and mineralization are performed by many organisms through the termination of phenolic aromatic rings. Phenolic compounds undergo oxidation with the aid of ring-dioxygenase producing dihydro diol in which it also produces dihydric phenol. Through the termination of ortho by catechol 2,3-dioxygenase enzyme, it produces cis-cis-muconate. In the termination of meta by catechol 2,3-dioxygenase enzyme, the catechol is changed and becomes semialdehyde muconate hydroxyl and other termination. Then the metabolite products are pyruvate acid, format acid, and acetaldehyde, which enter the Kreb cycle (Das and Chandran *et al.*, 2011).

Products of Kreb cycle are water and CO_2. Finally, the degradation process will produce H_2O, CO_2, and other compounds, which will be accumulated. Biodegradation consists of two or more steps, biotransformation, and/or mineralization. Biotransformation is completely basic of organic complex substance degradation yielding a simpler product. The biotransformation process is often accompanied by substrate cometabolism, which is used regularly by bacteria for growth and energy. The low percentage of a drop of TPH concentration as the impact of an addition of hydrocarbon-degrading bacteria might be caused by the degradation of hydrocarbon, which is still in the biotransformation stage. It means that the products of the biotransformation stage are only compounds that have not undergone degradation perfectly. Then those compounds will be mineralized by other bacteria that have the ability in decomposing hydrocarbon compound.

4.4 Bioremediation model

Data analysis reveals that both biomass and nitrogen concentration were in acceptable level in the treatment of combination of three organisms (PSB or hydrocarbon-degrading bacteria, mycorrhizae, and *Rhizobium*). The increase of phosphorus concentration was found in the treatment of combination of mycorrhizae and hydrocarbon-degrading bacteria or PSB. Furthermore, the decreasing of TPH concentration was found in the treatment of combination of hydrocarbon-degrading bacteria or phosphate-solubilizing bacteria and *Rhizobium*. In addition, a good status of plant biomass was indicated by the treatment of combination of three organisms. It is supported by the role of mycorrhizae to increase the availability of phosphorus, nitrogen, zinc, and other nutrients for plant growth and to increase the survival of plants in disadvantageous condition. These findings are relevant with the experiment on mycorrhiza-assisted phytoremediation that revealed that mycorrhizal fungi stimulate the activity of rhizosphere microorganisms. Fungus in the mycorrhizae will obtain the product of photosynthesis from plants. Meanwhile, the presence of *Rhizobium* can increase the availability of nitrogen in the medium of growth. Then the bacteria also will be able to obtain products of photosynthesis from plants. Other studies showed that the most promising approach was an application of plant–bacteria–mycorrhiza systems. Liu and Dalpe (2009) confirmed that anthracene and phenanthrene could be removed faster by supplying mixture of bacteria and culture of *G. versiforme* and *R. intraradices* because the mycorrhizae hyphae can secrete glomalin, a putative homolog of a heat shock protein 60 (HSP60) that stabilizes soil aggregates and increases the hydrophobicity of soil particles, what is important for the fate of PAHs in soil (Gadkar and Rillig, 2006; Auge, 2004). This protein may also influence the abundance of microorganisms. Even the glomalin may exceed soil microbial biomass by as much as 10–20-fold (Rillig *et al.*, 2001).

Furthermore, the problem of the condition of the medium of growth containing high hydrocarbon compounds can be solved by adding the hydrocarbon-degrading bacteria in the medium in order to decomposed hydrocarbon into a simpler form, which is signed by the low concentration of TPH. The addition of hydrocarbonoclastic bacteria and PSB also can support the role of mycorrhizae because these bacteria also can solubilize the phosphate. In this model, the initial stage was restoring the structure and texture of Lapindo mud by applying the appropriate composition between sand and mud. Furthermore, the hydrocarbon-degrading bacteria, which are also capable of solubilizing phosphate, were applied in the medium of growth in which it was expected that the land can be restored chemically. *Rhizobium* and mycorrhizae were also applied in order for the primary root of plants can interact with those organisms so the protection pattern of survival plants can be formed. Another finding reported that root penetration by the fungal hyphae can also be facilitated by some endophytic plant-growth-promoting rhizobacteria (PGPR) that excrete pectinases and cellulases (Yu *et al.*, 2011).

In general, a better understanding of plant–bacteria symbiosis could be exploited to enhance the remediation of hydrocarbon-contaminated soils in conjugation with sustainable production of crop for biomass. Plant and their associated bacteria interact with each other whereby plant supplies the bacteria with a special carbon source that stimulates the bacteria to degrade organic contaminant in the soil. Vice versa, plant-associated bacteria can stimulate their host plant to overcome hydrocarbon pollutants, to improve plant growth and development. In addition, benefits from their associated bacteria possessing hydrocarbon-generating potential are leading to enhanced hydrocarbon mineralization and lowering of both phytotoxicity and evapotranspiration of volatile hydrocarbons (Khan *et al.*, 2013). It seems that the effectiveness of mycorrhizae inoculation in the biodegradation of hydrocarbons, especially PAHs, was more stimulated when the mycorrhiza interacted with soil microorganisms because the application of mycorrhiza may increase the phytoremediation efficiency through increased biodegradative activity of

roots and rhizosphere microorganisms, improved adsorption and bioaccumulation of hydrocarbons by roots, and the enhancement of plant growth (Rajtor *et al.*, 2016).

The finding in this study confirmed that multicomponent phytoremediation systems are based on the synergetic effect in the plant–bacteria–mycorrhiza relationship where bacteria may act as "mycorrhizal helpers." Thus, the effectiveness of mycorrhiza inoculation in the biodegradation of hydrocarbons, especially PAHs, was more pronounced when the mycorrhiza cooperated with soil microorganisms. In this case were interactions with rhizobium, PSB, and hydrocarbon-degrading bacteria.

5. Conclusion, implication, and suggestion

The developed bioremediation system was based on the synergetic effect in the plant–bacteria–mycorrhiza cooperation. Multisymbiotic microorganism in the oil-contaminated soil played an important role in the transfer of energy and nutrient within the system including the growth stimulation. Thus, the effectiveness of mycorrhiza inoculation in the biodegradation of hydrocarbons, especially PAHs, was more stimulated when the mycorrhiza cooperated with soil microorganisms. Bioremediation model proposed, which is the analogy of tripartite symbiosis between plants, mycorrhizae, and *Rhizobium* but also adding plant growth bacteria such as PSB and hydrocarbon degradating bacteria, was proved effectively to increase the plant survival in oil-contaminated soil.

It is recommended that further study can be conducted directly in the field so the effect of multisymbiotic microorganism to improve the plant survival in oil-contaminated soil can be easily detected. Furthermore, the research can be focused to study the profile of protein and amino acids expressed by plants in stress condition in the view of the biomolecular aspect.

This research aimed to look for an information pattern of multisymbiotic relationship mechanisms in legume plants to survive in their environmental conditions. Multisymbiotic pattern will be applied in developing the model in the bioremediation process of Lapindo mud, which is also polluted with oil (rarely disclosed even though the TPH levels of the previous results were relatively high up to 41,000 ppm). The model will be developed using the benefits derived from hydrocarbon-degrading compounds, PSB, Rhizobium bacteria, and mycorrhizal fungi through soybean testing plants that have been proven applied in oil-contaminated areas (Rahayu *et al.*, 2010). Bioremediation model is expected to obtain through a multisymbiotic relationship pattern between organisms and each symbiont role so that plants tolerate the environmental conditions in Lapindo mud. The discovery of this bioremediation model can be used to determine biological agents that can repair Lapindo mud in terms of physics, chemistry, soil biology, so that it is feasible to be used as a planting medium for cultivated plants.

The results of this study should be used as a reference for using Lapindo mud as a planting medium. Future studies should be carried out for direct field trials to determine the effect of adding experimental organisms in increasing the effectiveness of soybean growing media. Moreover, the future direction of research will focus on the study of protein profiles and amino acids expressed when plants are under various environmental stresses and lead to biomolecular studies expected to contribute to the development of science and technology. For example, the study of amino acid proline is still controversial because of its role as an "amino acid marker" or "osmoprotectant" in stressed plants (Dobra *et al.*, 2010) considering that the amino acid proline is also in high concentrations during the generative phase (Mattioli *et al.*, 2009).

List of Abbreviations

TPHs	Total petroleum hydrocarbons
PAHs	Polycyclic aromatic hydrocarbons

PGPR	Plant-growth-promoting rhizobia
AMF	Arbuscular mycorrhizal fungi
BNT	Beda nyata terkecil
N	Nitrogen concentration
P	Phosphorus concentration
PGPR	Plant growth promoting rhizobacteria
PHCs	Petroleum hydrocarbon
M	Mycorrhizae (Glomus mosseae)
R	Rhizobium japonicum
B1	Pseudomonas pseudomallei
B2	Pseudomonas flourescens
B3	Pseudomonas stutzeri

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