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NITROGEN-FIXING BACTERIA INOCULATION (NFB) AND COMPOST APPLICATION IN PHYTOREMEDIATION OF PETROLEUM-CONTAMINATED SOILS USING RAMIE PLANT (Boehmeria Nivea L.)

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ABSTRACT

Phytoremediation is an alternative method to reduce toxic concentrations in hydrocarbon contaminated soils. The potential plant used in the phytoremediation of petroleum waste hydrocarbons is Ramie (Boehmeria nivea L.) plants. Efforts for improving the Ramie performance in the phytoremediation process of petroleum need to be done through inoculation of N-fixing Bacteria (NfB) such as Azotobacter sp. and Azospirillium sp., and also Cow manure compost application. Factorial randomized block design was used as a treatment design. The first factor was the inoculation of NfB: control; mixed culture of NfB; Azospirillum sp. and Azotobacter sp. and the second factor was the application of compost at the level of: control; 5 t / h; and 10 t / h. The results showed that inoculation of NfB mixed culture decreased the Azotobacter spp population, but did not affect to the Azospirillium spp. population. The compost application at a dose of 10 t/ h could significantly increase the Azotobacter spp. population. The treatment of NfB mixed culture and compost application at a dose of 5 t/ h significantly increase the efficiency of hydrocarbon biodegradation, the efficiency of hydrocarbon biodegradation values were of 83.1% and 83.0%, respectively. Ramie plant which interacted with the NfB indigenous was able to degrade petroleum hydrocarbons with biodegradation efficiency values in the values range of 80.0 - 80.9%. Inoculation of NfB mixed culture was able to function as a bio-stimulant for increasing the biodegradation efficiency of petroleum hydrocarbons in the phytoremediation system by using Ramie plants

Keywords: Azospirillium sp., Azotobacter sp., Biodegradation, Phytoremediation, Hydrocarbon..

1. INTRODUCTION

Indonesia is one of petroleum-producing countries. According to data from the Central Statistics Agency [2] reported that oil and condensate production in Indonesia in 2018 reach 281 826.61 barrels/year, and petroleum is dominantly consisted by hydrocarbon compound. From each barrel of crude oil that is explored and processed into various energy raw material release side products and waste that will be generated continuously and accumulated, so that it can pollute

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the environment that requires comprehensive handling. The right choice for Biological waste treatment that can be done is by phytoremediation method, because Phytoremediation has advantages over other waste treatment technologies, which are natural and environmentally friendly processes, lower costs, permanent reduction of organic matter, a synergy relationship between the environment, organisms, and plants [15]. Plants that can be used for phytoremediation are the plant that can accumulate or degrade pollutants in a polluted environment. One of the plants that could potentially be used is Ramie (*Boehmeria nivea* L.), is a fiber-producing plant that has fairly high stem biomass, and plant-disturbing organisms, and is also easily cultivated, and has a strong root system [17], tolerant of various extreme environments such as drought, infertility, disease, pest and heavy metal attacks [19]. And [21] reported that Ramie plants were able to degrade 7% Total Petroleum Hydrocarbon (TPH) petroleum waste with a hydrocarbon degradation efficiency of 75.07%. However, efforts to improve the performance of Ramie plants as phytoremediation plants for removing petroleum contaminated soil still need to be done. One strategy that can be done is through the inoculation of N-fixing bacteria and the aplication of the right organic matter.

Nitrogen-fixing Bacteria (NfB) used in this study were *Azospirillum* sp. and *Azotobacter* sp., while the organic material used is cow dung compost. Both components are expected to function for stimulating the phytoremediation process of hydrocarbon compounds by Ramie plants. *Azospirillum* sp. and *Azotobacter* sp. are classified in the category of plant growth-promoting rhizobacteria (PGPR) which can synthesize growth hormones [3]. According to the study [21] that the inoculation of *Pseudomonas cepacia* and 2% *Azotobacter* sp. in phytoremediation of petroleum using Ramie plant showed a hydrocarbon degradation efficiency of 88.43%. That is because these microbes produce phytohormone which can increase the growth of Ramie plants. *Azospirillum* sp. and *Azotobacter* sp., play a role to stimulate plant growth and formation of Ramie plant root exudates. *Azospirillum* sp. can interact with the roots of various plants, able to overhaul organic cellulose and organic material consisted by fats and proteins, able to fixing di-nitrogen and dissolve phosphate and synthesize IAA plant growth hormone [8],[22], and IAA hormones produced by *Azospirillum* sp. trigger cell division and propagation [10]. While *Azotobacter* sp. can synthesize phytohormones and B-vitamins in the form of biotin [13] and also fixation of N_2 transformed into Ammonium which will be used for Ramie plants nutrition. The nitrogen is an element that plays an important role in the formation of chlorophyll [14] for photosynthesis process. As a result of stimulation of the two N-fixing bacteria will increase root growth and increase root exudates production. And root exudates produced from Ramie plants will be used as a source of nutrition and induction of petroleum degrading bacteria in soil [21].

Organic manure are all waste products from livestock that can be used to add nutrients, improve physical properties, and soil biology [5]. The type of organic manure that is widely used by the community is cow manure. According to [16] reported that the nutrient content contained in cow dung fertilizer is nitrogen at 0.4%, phosphorus 0.2%, and potassium 0.1%. Amendment cow manure compost can provide macro (nitrogen, phosphorus, potassium, calcium, and sulfur) and microelements (iron, zinc, boron, cobalt, and molybdenum), increase soil microbiological activity, increase cation exchange capacity, improve soil structure, and increasing water resistance. According to [11] reported that the effect of adding organic nutrients to the bioremediation of crude oil spills in the East Surabaya coastal environment can reduce

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hydrocarbon concentrations by 88.25% within 30 days. High porosity will increase oxygen exchange in the soil. Sufficient oxygen supply allows microbes to work optimally to degrade pollutants.

This research is focused on examining the effectiveness of exogenous N-fixing bacteria (*Azospirillum* sp. and *Azotobacter* sp.) inoculated and the application of cow dung compost for enhancing the growth of Ramie plants (Phytoremediator), adaptability of *Azospirillum* sp. and *Azotobacter* sp., and the biodegradation efficiency of oil hydrocarbons in the phytoremediation process by the Ramie plant.

2. MATERIAL AND METHOD

The experiment was carried out at the Greenhouse of the Faculty of Agriculture, Padjadjaran University, Jatinangor, Sumedang Regency, West Java Province height of about 700 meters above sea level.

2.1. Materials and Method

The materials used in this study consist of Inceptisol - Ciparanje Jatinangor soil, crude oil waste from PT. Pertamina RU VI Balongan Indramayu - West Java contains a TPH concentration of 92.5%, N-fixing bacterial inoculants (*Azotobacter* sp. and *Azospirillum* sp.) with a density of 10^6 CFU / ml. The Ramie plant clones used were the rhizome part with the Lembang variety. *Azotobacter* sp. was cultured on Ashby media (KH2PO4; MgSO4.7H2O; Mannitol; NaCl; CaCo3; Agar), and NFB (Nitrogen Free, Bromthymol Blue) media (Malic Acid; KOH; K2HPO4; FeSO4. & H2O; MnSO4.7H2O; MgSO4.7H2O); CaCl2; N2MoO2; BTB (0.5% in 95% alcohol); Bacto agar; pH) for *Azospirillum* sp. culturing, and Cow dung compost.

The study was conducted on a greenhouse (microcosmic) scale using a factorial randomized block design.

The first factor (A) is a consortium of nitrogen-fixing bacteria consisting of four levels, namely:

a0: Without Inoculants (control)

a1: Mixed culture of nitrogen-fixing bacteria (*Azotobacter* sp. And *Azospirillum* sp.)

a2: *Azospirillum* sp.

a3: *Azotobacte*r sp.

The second factor (B) is compost dosage which consists of three levels, namely:

b0: Without Compost aplication

b1: Compost 5 t / ha

b2: Compost 10 t / ha

2.2. Organic Compost Preparation and Description of Nutrient Content.

In this study, the type of organic material used came from the compost of cow dung. According to the Tobacco and Fiber Crops Research Institute, Malang Agricultural Research and Development that the need organic material for Ramie plant is 10 t/ha.

2.3. Ramie's Nursery Preparations and Preparation of petroleum-contaminated soil and Ramie planting

Ramie plants used in his study was 12-week-old seedlings (180 nursery days). The nursery material was taken from the adult Ramie plant rhizome which can already be used for breeding. The Ramie clones used was var. Lembang clones. The size of Ramie seedlings planted was ± 10 cm with the number of 5-7 leaves. Three quarters (3/4) parts of the plant were immersed in the soil media and a quarter (1/4) part was on the ground surface. Stems of Ramie seedlings were tilted 45%. The nursery media are soil and compost (1: 1 ratio).

Jatinangor's Inceptisol soil was contaminated with crude oil homogeneously with a total Petroleum Hydrocarbon (TPH) load of 7%. Each test pot contains 5 kg of contaminated soil.

2.4. Inoculation of Nitrogen-fixing Bacteria Consortium and Ramie Plants

Nitrogen-fixing bacterial (BPN) were inoculated according to the treatment dose into the soil that has been mixed with organic material and contaminated by petroleum. While the inoculation of *Azotobacter* sp. and *Azospirilium* sp. culture were by pouring the NfB culture around the rooting area around 4%/ TPH loading or as much as 15.12 ml with a population density of 10^6 CFU / g soil. In this study, inorganic fertilizers use N, P, K compound fertilizer, with 1/2 recommended dosage, so the fertilizer requirement per polybag was 0.21875 g Urea, 0.025 g SP36, 0.075 g KCl. Fertilizer application was done once after a week of planting by spreading in a run around the roots of Ramie plant spacing ± 5 cm.

2.5. Maintenance and Observation

Maintenance of Ramie plants in this study includes watering, weeding, and controlling plant pests (OPT). Watering plants is done every day by maintaining field capacity. Observations made include analysis of the efficiency of hydrocarbon degradation, the population of *Azospirillum* sp., and *Azotobacter* sp. by using the TPC (*Total plate count*) method, an analysis of the dry weight of Ramie plants was carried out at the end of the observation.

3. RESULT AND DISCUSSION

3.1. The population of Nitrogen-fixing Bacteria at the end of the phytoremediation process.

Inoculation of *Azotobacter* sp. and *Azospirillium* sp. (N-fixing bacteria) and compost did not show any interaction effect. The mean values of bacterial populations at each level of the treatment factor are presented in Table 1.

Table 1. Effect of NfB Inoculation and Compost application on Total Bacteria Population in Phytoremediation Process of Petroleum Hydrocarbons during 10 Weeks (end of observation)

Treatment	**Bacteria Population ($x10^7$ CFU/ml)**	
	***Azospirillum* spp.**	***Azotobacter* spp.**
Nitrogen-fixing bacteria (A)		
a_0 : Without Nitrogen-fixing Bacteria (control)	52.1 a	50.2 b
a_1 : Mix culture of NfB (*Azotobacter* sp. and *Azospirillum* sp.)	37.7 a	34.6 a
a_2 : *Azospirillum* sp.	42.2 a	36.6 a
a_3 : *Azotobacter* sp.	39.0 a	48.7 b
Compost (B)		
b_0 : Without Kompos	43.3 a	36.6 a
b_1 : Compost 5 t/ha	44.9 a	38.7 a
b_2 : Compost 10 t/ha	40.1 a	52.3 b

Note: The average value of the treatment followed by the same letter indicates the treatment factor did not significantly affect the response based on analysis of variance at the 5% level.

The population of Azotobacteri spp. significantly decreased due to the inoculation of *Azospirillium* sp. and mix culture of *Azotobacter* sp. and *Azospirillium* sp. This investigation showed that *Azospirillium* sp. used in this study more directly suppressed the growth of *Azotobacter* spp. It can be seen from the population of *Azotobacter* spp. due to the presence of *Azospirillium* sp. tend to be the lowest.

The results of initial soil analysis of the average total population of *Azospirillum* spp. indigenous was 9.9 x 10^7 CFU / g. In general, it shows that the land inhabited by Rami can increase the population of *Azosprillium* spp. and *Azotobacter* spp. This occurs due to the root exudate which is excreted by Ramie plant, and roots acted as a source of nutrition and carbon source for *Azotobacter* spp. and *Azospirillium* spp. in rhizosphere area. However, by inoculation of *Azospirillum* sp. and *Azotobacter* sp. have not been able to increase the population of *Azotobacter* spp. and *Azospirillium* spp. in experimental soil. Thus the addition of mixed culture of *Azotobacter* sp. and *Azospirillum* sp. in oil-polluted soils not yet considered optimal. NfB inoculants application with a population density of 10^6 CFU/ ml is considered too smaller when compared to the indigenous bacteria present in soil. It is recommended to increase the density of the NfB population used, so that it can function as expected.

Azotobacter sp. inoculation treatment showed a significantly increased in the *Azotobacter* spp. population compared to the treatment with *Azospirillium* sp., this phenomenon shows that the addition of inoculant *Azotobacter* sp. exogenous was able to colonize the rhizosphere of Ramie. And Azotobacter sp. inoculated as a bio-fertilizer acted mobilization to the rhizosphere which allows bacteria to multiply and received the sources of nutrients from root exudates [6]. Thus the application of *Azotobacter* sp. was able to provide the desired effect as Plant Growth Promoting Rhizobacteria (PGPR) producing IAA (Indole Acetic Acid) hormone and potentially being a biofertilizer agent for N and P sources .

Effect of compost on a total population of *Azotobacter* sp. showed that the level of compost treatment of 10 t/ ha showed a higher value and significantly different compared to the treatment without compost, and compost treatment at a dose of 5 t / ha singly. Addition of compost treatment of 10 t / ha can supply the needs of carbon sources and nutrients needed by *Azotobacter* spp, so that under conditions of nutrient adequacy can spur increased populations of *Azotobacte*r sp. Almost 50% of the cell dry weight consists of carbon, therefore carbon is the most important macronutrient needed [9]. The effect due to the application of cow manure to the population of *Azospirillium* spp. did not show significant differences between each compost dosage application.

The compost application of cow dung up to 10 t / h has not been able to increase the population of *Azospirillium* spp. This illustrates that *Azospirillium* spp requires a higher source of carbon and nutrients for their growth. compared with the needed carbon source for *Azotobacter* spp. growth, so that resulting in a limited carbon source for *Azospirilliu*m spp. The limited Carbon source is an inhibiting factor for *Azospirillum* sp. growth and also due to competition with other microbes for the needs of carbon and N sources.

3.2. Dry Weight of Ramie Plant after 10 weeks planting

Ramie plant growth is represented by the value of dry weight of plant at the end of the observation (10 weeks), showing that NfB inoculation and compost application did not show significantly different results in each treatment. The average values of dry weight of Ramie plants at each level of the treatment factors are presented in Table 2.

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Table 2. Effects of application of Inoculants and Compost on Dry Weights of Plants on the Phytoremediation Process of Petroleum Hydrocarbons during 10 Weeks

	Dry Weights of Plants (g)
Nitrogen-fixing bacteria (A)	
a_0 : Without Nitrogen-fixing Bacteria (control)	40.5 a
a_1 : Mixed culture of NfB (*Azotobacter* sp. dan *Azospirillum* sp.)	46.7 a
a_2 : *Azospirillum* sp.	45.4 a
a_3 : *Azotobacter* sp.	43.5 a
Compost (B)	
b_0 : Without Kompos	46.0 a
b_1 : Compost 5 t/ha	41.0 a
b_2 : Compost 10 t/ha	47.5 a

Noted: The average value of the treatment followed by the same letter indicates the treatment factor did not significantly affect the response based on analysis of variance at the 5% level

The application of NfB and compost at several levels did not have a significantly different effect on the dry weight of Ramie plants. In line with observations of *Azotobacter* sp. and *Azospirillum* sp. inoculation, showed that the exogenous bacterial inoculation has not been able to colonize the roots of Ramie plant, so that the application of NfB inoculant also has not been able to increase the dry weight of Ramie plants compared to control. However, the inoculation of Azotobacter sp., *Azospirillium* sp, and both inoculant mixtures appear to have the potential to increase the dry weight of the Ramie plant compared to the control even though it is not significant. This result showed that the possible inoculant density of *Azotobacter* sp. and *Azospirillium* sp. inoculated were not enough to act as biostimulants in significantly increasing the dry weight of Ramie plant. Although according to [4] research that *Azotobacter* sp. and *Azospirillum* sp. is a group of the genus of bacteria that can synthesize PGPR (plant growth-promoting rhizobacteria) and growth hormone. However, in this study, the role of both inoculants as PGPR was not maximal performance. Likewise, the compost application that functions as a provider of macro and micronutrients for Ramie's plants also has not been able to increase the dry weight of Ramie plants. However, the function of adding compost to oil-contaminated soils is to increase porosity and soil aeration, so that oxygen demand for hydrocarbon integration microbes can be fulfilled.

3.3. The efficiency of Hydrocarbon Degradation

The inoculation of NfB and compost application also showed no interaction effect, but both factors showed the existence of an independent influence on the efficiency of hydrocarbon degradation. The average value of hydrocarbon degradation efficiency at each level of the treatment is presented in Table 3.

Table3. Effect of NfB Inoculant and Compost Applications on the Efficiency of Hydrocarbon Degradation in the Phytoremediation Process of Petroleum Hydrocarbons for 10 Weeks

	The Efficiency of Hydrocarbon Degradation (%)
Nitrogen-fixing bacteria (A)	
a_0 : Without Nitrogen-fixing Bacteria (control)	80.0 a
a_1 : Mixed culture of NfB (*Azotobacter* sp. dan *Azospirillum* sp.)	83.1 b
a_2 : *Azospirillum* sp.	71.3 a
a_3 : *Azotobacter* sp.	80.1 a
Compost (B)	
b_0 : Without Kompos	80.9 a
b_1 : Compost 5 t/ha	83.0 b
b_2 : Compost 10 t/ha	72.0 a

Noted: The average value of the treatment followed by the same letter indicates the treatment factor did not significantly affect the response based on analysis of variance at the 5% level.

Table 3. shows that the effect of Nfb inoculant on the efficiency of hydrocarbon degradation showed that treatment of the mixed culture of *Azotobacter* sp. and *Azospirillum* sp. was resulting in higher average biodegradation efficiency of petroleum hydrocarbon, and significantly different compared to the control and to the mixe culture treatments. The function of adding exogenous inoculants of the NfB mix culture as Plant Growth Promoting Rhizobacteria (PGPR) can exert the expected effect. *Azotobacter* sp. is producing IAA (Indole Acetic Acid) hormone and potentially as a biological agent supplying N and P. While *Azospirillum* sp. can produce IAA and as a supplier of these phytohormones for plant growth as well as root development [12]. So the results of this study indicate that there is an increase in the efficiency of biodegradation of petroleum hydrocarbons if *Azotobacter* sp and *Azospirillium* sp. used in a mixture which

resulting efficiency of biodegradation of petroleum hydrocarbons was 83.1%. This happens because of a mixture of *Azotobacter* sp. and *Azospirillium* sp. culture can contribute to the availability of nutrients and phytohormones needed by Ramie plants, so that it will optimize the development of roots and the formation of root exudates which will be used to increase population and stimulate petrophilic microbial activities to degrade petroleum hydrocarbons. Reported by [21] that root exudates have an important role in providing nutrition and stimulating microbial activity to degrade indigenous hydrocarbons. And investigation resulted by [4] showed that *Azospirillum* sp. was able to use hydrocarbons as carbon sources and energy sources. While [21] reported that the role of *Azotobacter* sp. apart from being a producer of phytohormones and N fixers, can also produce extracellular compounds that function as biosurfactants. Biosurfactant can increase the solubility of hydrocarbons and increase the availability of substrate, namely oil hydrocarbons which are large droplet oil size into micell oil size that is easily used by Petrophylic microbes.

The effect of compost application on the efficiency of hydrocarbon degradation showed that the compost treatment on the level of 5 t / ha resulted in a higher value of the efficiency of hydrocarbon degradation significantly different than the treatment without compost and compost 10 t / h treatment. This resulting figures the application compost at the right dose can improve soil properties and contain essential mineral nutrients for plants [20]. The addition of the adequate dose of compost can to enlarge the porosity of soil contaminated with petroleum, which resulting in High porosity will be increasing oxygen exchange in the soil, and oxygen adequate in the soil is the important factor for hydrocarbon biodegradation process. According to [7] and [1] reported that the right oxygen supply in the soil system allows microbes to work optimally in degrading pollutants, and optimally aeration can support the growth of microbes in the soil.

4. CONCLUSION

Azospirillium sp. inoculation can reduce the population of *Azotobacter* spp. in petroleum contaminated soil during 10 weeks after Ramie planting, while the population of *Azospirillium* spp. was not affected by the inoculation of NfB. A compost dose 10 t / h amendment can increase the population of *Azotobacter* spp., but has not been able to increase the population of *Azospirillium* spp. While the inoculation of *Azotobacter* sp., *Azospirilium* sp. and Compost application at a dose of 10 t/ h showed the potential to increase the dry weight of Ramie plants, although not yet significant. The treatment of NfB mixed culture can improve the efficiency of biodegradation of petroleum hydrocarbons significantly, higher than control which results in an efficiency value was 83.10%. And also the application of compost dose of 5 t/ h has shown a significant increase to the efficiency of biodegradation of petroleum hydrocarbons, higher compared to the control treatment and 10 t/ h dose treatment. The potential of indigenous NfB to the hydrocarbon biodegradation efficiency using Ramie plant showed in the range of 80.0 - 80.9%. The density of *Azotobacter* spp. indigenous was in range 36.6. 10^7 - 50.2. 10^7 CFU/ g of soil, and density of *Azospirillium* spp. indigenous was in range 43.3. 10^7 - 52.1. 10^7 CFU / g of soil. Those NfB density was potentially for inducing hydrocarbon biodegradation in Phytoremediation system by using Ramie plant.

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