Potential of *Boehmia nivea* as phytoremediator for petroleum-contaminated soil following nitrogen-fixing bacteria inoculation

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Abstract. Phytoremediation is one of an alternative technology to overcome a petroleum-contaminated soil. *Boehmia nivea* or Ramie has the potential to be used as a phytoremediator agent to degrade petroleum hydrocarbons. The effort to improve the performance of Ramie in degrading petroleum hydrocarbons is through the concept of phytostimulant inoculation in biological fertilizer. The objective of pot experiment was to investigate the performance of Ramie as a phytoremediator of petroleum waste. The research was designed in a randomized block design and consisted of Ramie treatment without inoculation, Ramie inoculated with 2% *Azotobacter vinelandii*, 2% *Azospirillum* sp., and 2% consortia of both inoculants. Experimental result showed that Ramie plants removed petroleum hydrocarbons up to 83.22% after 13 week of inoculation. The efficacy of hydrocarbon degradation increased significantly if Ramie plants was inoculated by N-fixing bacteria, but the biodegradation efficiency in biofertilizer treatments was not significantly different.

1. Introduction

Petroleum is still one of the biggest energy sources in the world. It is formed from the decomposition of plant and animal fossils over millions of years; and contains various chemical compounds such as vanadium, nickel, sodium, iron, aluminium, calcium, magnesium and hydrocarbons [4]. According to Indonesian Statistical Bureau (2015), fossil fuel consumption is estimated to reach 1.6 million barrels per day. This resulted in 33% of potential waste that requires proper treatment. Phytoremediation is one of the viable method to treat hydrocarbon contamination through the utilization of remediator plants. This method is simple, efficient, cost effective, and environmentally friendly [11]. Certain plants can survive in high concentrations of pollution [5] one of them is Ramie (*Boehmerianivea* (L.) Gaud). Ramie is an annual fiber plant that is easy to be cultivated and can reach 200-250 cm in heights, high biomass, and can adapt well to unfavourable growth conditions [13]. Ramie as a remediator plant for petroleum has not yet studied intensively, but previous research has shown that it was and economically ideal plant for areas polluted by organic pollutants [16,17]. Petroleum hydrocarbons are classified as toxic and difficult to degrade. Certain level of Total Petroleum Hydrocarbon (TPH) in petroleum can reduce the population of most soil microbes when soil contaminated with it [9]. Organic and inorganic contaminants found in the soil can be overhauled in various ways namely phytodegradation, phytostabilization, phytoevaporation, phyto-extraction,
phytostimulation and rhizodegradation [6]. Organic pollutants were completely degraded into relatively non-toxic elements such as CO₂, nitrate, chlorine and ammonia with phytoremediation method [7]. Remediator plant degrades pollution from its rooting system, by stimulating the growth and activity of microbes contaminants to degrade or reduce the toxicity of an organic or inorganic compounds [11].

Microorganisms can assist the remediator plant degradation process of hydrocarbons. The difficulties of hydrocarbon petroleum to dissolved in water might be overcome by application of biosurfactants-producing microbes [1]. Some bacteria that play a role as biosurfkatan producer and biofertilizer are Azotobacter sp., Azospirillum. Azotobacter is a bacterium that can fixate nitrogen (N₂) and acts as a plant growth promoting rhizobacteria (PGPR) through the production of growth hormones such as Indole Acetic Acid (IAA). Azotobacter chroococcum produce extracellular polymeric substances consisted of polysaccharides and fatty acids that can act as biosurfactant and increase the biodegradation of petroleum waste [14]. Azospirillum sp. fixes nitrogen and produce growth hormones that cause the root to extend and expand [8]. This research is focused on the study of the potential of Ramie plant as a remediator and application of N-fixing inoculants to improve biodegradation performance.

2. Materials and methods

Inceptisols soil order of Jatinangor phytoremediation media was taken by composite method from the 0-20 cm depth. Waste petroleum used in the form of crude oil from Kelayan Pertamina, Cirebon. The soil acidity was 6.37, C-Organic was 1.26% (very low) total-N is 0.26% (medium), C/N ratio was 5 (low), and P₂O₅ Bray I was 9.9 ppm P (medium).

Two kilograms petroleum contaminated soil was put into a pot, 30 cm in height and 25 cm wide. Petroleum used contained 7% of total petroleum hydrocarbon (TPH). Inoculants (2% per waste load, w/w) were applied to the contaminated soil for each species. Bacterial inoculants were obtained from the collection of Soil Biology Laboratory, Department of Soil Science, Universitas Padjadjaran. The Ramie plant used in this experiment was obtained from Universitas Padjadjaran experimental greenhouse collection and was vegetative propagation from 21-year-old rhizoma cuttings.

The experimental design used was a Randomized Block Design consisting of four treatments: i₀ = Control (without giving inoculants); i₁ = Azotobacter vinelandii liquid culture at a dose of 2% of waste load; i₂ = Azospirillum sp. liquid culture at a dose of 2% of waste load; i₃ = Mix culture (Azotobacter vinelandii, Azospirillum sp.), at a dose of 2% of waste load. Each treatment was repeated three times.

Preparation of Contaminated soil and Application of Inoculant Types: The soil used in the experiment came from topsoil (0-20 cm). Two kilograms of treated soil was inserted into a 30x25x25 cm pot. Petroleum contaminated soil contained 7% of total petroleum hydrocarbon per kilogram soil. Inoculants applied to petroleum-contaminated soil for each species were 2% per waste load (w/w) of TPH concentration.

3. Results and Discussion

3.1. Efficiency of Hydrocarbon Degradation

Efficiency of hydrocarbon degradation is presented in table 1. Different N-fixing bacteria inoculation resulted in significant degradation efficiency values compared to the control. Both individual and consortia of inoculant hydrocarbon degradation efficiency values differences are negligible, but significantly different than that of control.
Table 1. Effect of Inoculants type on the Efficiency of Hydrocarbon Degradation during 13 week incubation.

| Treatment                                    | Degradation Efficiency(%) |
|----------------------------------------------|----------------------------|
| i0 = Control                                 | 83.22a                     |
| i1 = Azotobacter vinelandii 2%               | 88.80b                     |
| i2 = Azospirillum sp. 2%                    | 88.48b                     |
| i3 = Consortia of Azotobacter vinelandii and Azospirillum sp., 2% | 88.98b                     |

The average treatment value marked with the same letter is not significantly different according to Duncan's Advanced Test at 5% significance level.

Azotobacter or Azospirillum as plant growth promoting bacterium may link this result to root growth stimulation. Biodegradation of petroleum hydrocarbons requires microorganisms that can produce oxidizing enzymes of petroleum hydrocarbons and can produce emulsifiers for stimulating hydrocarbon biodegradation process [2]. *Azotobacter vinelandii* and *Azospirillum* sp. provides nitrogen, essential nutrient for root growth. Extensive rooting system resulted high exudates production, which may contribute to petroleum degradation process. Both bacteria shown beneficial synergism, where there is a synergism between inoculants that are applied to degrade hydrocarbons.

Inoculating an inoculant mix culture can accelerate the process of soil remediation because inoculants can produce bio surfactants. In this research, there is an inoculants mix culture, there is *Azotobacter vinelandii* which can produce bio surfactants. According to [15], Biosurfactants which function to convert petroleum large droplet oil into micelle oil, so that they are more easily broken down by petrophilic bacteria which results in faster biodegradation rates. In this study shows that the efficiency of biodegradation due to mix culture treatment reached 88.98%.

3.2. Density of *Azotobacter* spp. during Petroleum hydrocarbon Biodegradation process

The treatment of N fixing bacteria inoculants did not significantly affect the density of *Azotobacter* sp. in ramie rhizosphere (Table 2). The control treatment resulted *Azotobacter* sp. dencity which higher than other treatment.

The effect of *Azotobacter vinelandii* treatment on total *Azotobacter* density in soil did not show an increase in *Azotobacter* spp. population. These conditions also indicate that the application of *Azotobacter vinelandii* is not able to increase the total *Azotobacter* population in the soil. While the treatment of *Azospirillum* sp. and a mix culture (*Azotobacter vinelandii, Azospirillum* sp.) showed the population value of *Azotobacter* sp. which tends to be the lowest compared with other treatments. But After growing rami (with or without bacterial inoculated) during 13 week resulted *Azotobacter* density increased almost 3 log (10 log). N fixing bacteria inoculation did not encrease the population *Azotobacter* compared to non inoculated one. It means that the rhizosphere of ramie enable to increase *Azotobacter* population. But Exogenous *Azotobacter* did not affect *Azotobacter* population after experiment.

Table 2. Effect of Inoculants N-fixing bacteria on *Azotobacter* spp. dencity in the Ramie rhizosphere during 13 week incubation

| Treatment                                    | Azotobacter spp. at the end incubation (1x10⁶cfu/g) |
|----------------------------------------------|-----------------------------------------------------|
| i0 = Control                                 | 19.20                                               |
| i1 = Azotobacter vinelandii 2%               | 19.12                                               |
| i2 = Azospirillum sp. 2%                     | 15.98                                               |
| i3 = Consortia of Azotobacter vinelandii and Azospirillum sp., 2% | 14.48                                               |
Azotobacter population at the end of the experiment increased for each treatment but the end of the experiments did not significantly different compared to control treatment. From this study it is evident that the density of Azotobacter at the beginning was only $4 \times 10^4$ cfu/g of land increasing to $19.2 \times 10^6$ cfu/g of soil. This indicates that initial Azotobacter in this experiment was able to adapt well to soil conditions that contain petroleum hydrocarbons.

3.3 Total Population of Azospirillum spp. during Petroleum hydrocarbon Biodegradation process

Inoculation did not show any effect on the total population of Azospirillum spp, compared to the control (Table 3). The total Azospirillum initial density of the soil before the experiment was $17 \times 10^4$ cfu/g and increased to $16.50 \times 10^5$ cfu/g in the control treatment after the experiment took place in week 13. Irrespective of statistical analysis, population of Azospirillum after inoculation if N fixing bacteria was higher than the control treatment. The experiment showed that inoculaion of Azotobacter, Azospirillum and its mixed culture might induce the growth of Azospirillum in petroleum-contaminated soils.

Table 3. Effect of N-fixing inoculants on the total population of Azospirillum spp. in the Ramie Rizosphere during 13 week Ramie Planting

| N-fixing bacterial Inoculants | Azospirillum sp. at the end of incubation in soil $(10^5\text{ cfu/g soil})$ |
|------------------------------|--------------------------------------------------------------------------|
| i0 Control (ramie without inoculants) | 16.50 |
| i1 Azotobacter vinelandii 2% dosage | 21.67 |
| i2 Azospirillum sp. 2% dosage | 18.50 |
| i3 Mix culture (Azotobacter vinelandii, Azospirillum sp.) 2% dosage | 20.67 |

Azospirillum density at the beginning and the end of the experiment also increased, both in the control treatment and other treatments. The addition of Azotobacter, Azospirillum, and mix culture inoculants might increase the total Azospirillum population even though the increase was not significant compared to the control treatment. This shows that the inoculation of exogenous inoculants has the potential to increase the proliferation of Azospirillum-cell in the soil. Thus Azospirillum has a better ability to use its existing carbon sources from the root exudates of ramie if it adds exogenous Azotobacter.

4. Conclusion

The efficiency of hydrocarbon biodegradation in the Phytoremediation system using Ramie plants inoculated with N-fixer bacteria showed significantly higher compared to uninoculated treatments. However, the biodegradation efficiency was not significantly different between the N-fixer inoculation. The potential of Ramie plant as a phytoremediation agent for petroleum hydrocarbons biodegradation at a waste load of 7% TPH showed the value of biodegradation efficiency of 83.22%. Azospirillum tends able compete for carbon sources derived from hydrocarbons from petroleum for its growth compared with Azotobacter in the petroleum contaminated soil and can effectively used the root exudates materials. The adaptability of initial inoculants and Ramie plant have the potential to remove petroleum hydrocarbon was reaching 83.22 % of biodegradation efficiency.
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