The potential of the mushroom log waste (MLW) and *Azotobactervinelandii* to improve hydrocarbon biodegradation for rehabilitation of petroleum contaminated soil

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Abstract. Bioremediation is the biological technology for removing toxic and complex pollutants in soil and water system by degrading or transforming the pollutants becoming harmless compounds. The research focused on determining the Mushroom log waste (MLW) and *Azotobactervinelandii* effect to improve the rate and efficiency of petroleum degradation in soil and assessment of functional microbial existing in that soil system. The randomized block design (RBD) was used with six treatments and three replications consisted of: Control; *A. vinelandii*; Petrophylic; Mushroom log waste (MLW); *A. vinelandii* + MLW; Petrophylic + MLW. The result showed that *A. vinelandii* treatment accelerated the hydrocarbon degradation rate from 0.007811 day⁻¹ to 0.011038 day⁻¹. While the hydrocarbons degradation rate by MLW treatment reached 0.026354 day⁻¹ with a biodegradation efficiency of 81.716 %. The MLW also enhanced bacterial petrophylic growth rate from 0.263029 day⁻¹ to 0.357115 day⁻¹, while *Azotobacter* spp. growth rate enhanced from 0.057886 day⁻¹ to 0.250719 day⁻¹. The MLW has potential used for supporting soil rehabilitation of petroleum waste, also increased existing of *Azotobacter* spp. population.

1. Introduction

The most demanded energy resource is Petroleum oil, still as a prime fundamental aspect which holds a role in running almost in industrial countries. Petroleum oil as an energy resource necessitates standard guidelines to be regarded for use in all of countries. Progressive economic growth of countries has been increasing for petroleum oil demand, which must be made effort by new exploration and production technologies [1]. But on the other side, the petroleum industry is responsible for generating high amounts of organic residues, as well as for the pollution of soils, rivers and seas.

The petroleum residues waste in Indonesia reached 1.380 barrels in 2010 and increased to 2.071 barrels in 2013. Petroleum Waste can contaminate soil and water up to the sub-surface and ground water aquifer layers [2], and that can damage an environment, soil, rivers and ground water. These problems resulted in less land safe for agriculture and specifically that hydrocarbons from crude oil spill for the plant can interfere to the seed germination, and it causes abnormality of cell growth of a
plant. So, it forces us to look for the proper technology and environmentally friendly to restore land damaged which caused by contaminated petroleum into a soil more productive and safe to use for media growth of crops, are also safe for the human environment.

The friendly technology for removing Hydrocarbon pollutant in the soil is bioremediation technology. Bioremediation use naturally-occurring microbes to transform toxic wastes into (normally) less toxic materials or even nontoxic materials [3, 4]. Bioremediation has been used extensively for many years as a successful method of treatment for hydrocarbon-contaminated waste [5]. One of the efforts which can be done to overcome the above problems is through technological innovation with the concept bioremediation stimulation and bioaugmentation system, namely by utilizing the potential of organic materials for increasing aeration level on the system and using bioagent double function [6, 7]. The concept of stimulation system in petroleum contaminated soil remediation are using stimulation materials which is by applying properly nutrients, aeration and optimally physical condition, and applying the proper organic material, while the bio-augmentation system is the method of bioremediation by injected specialized microbial inoculants into the contaminated zone. Aeration improvement might be done by turning and adding organic matter, or by combining these practices. The addition of organic matter to the hydrocarbon contaminated soil can be beneficial as it is a source of co-substrates, nutrients and microorganisms, and ameliorates the structure and water retaining capacity of the soil [8].

Biodegradation process is one of the primary mechanisms to eliminate hydrocarbon petroleum and other hydrocarbon pollutants from the environment [9]. One of the potential organic material that can be used is a waste of mushroom log waste and application *Azotobacter vinelandii*. The mushroom log waste (MLW) can be functioned as a bulking agent which is able to improve aeration in the soil system. Meanwhile, *A. vinelandii* is a rhizobacteria which has been known having a sophisticated character as a producer of biosurfactant compounds [10] and was proven to stimulate the occurrence of hydrocarbon degradation process faster and more efficient than without *A. vinelandii* application [11].

The potentially of the microorganisms pointed out in literature as agents of degradation of several compounds indicates biological treatments as the most promising alternative to reduce the environmental impact caused by petroleum oil spills. Microbial inoculant can be utilized to improve the rate of hydrocarbon biodegradation in the process of bioremediation. The inoculants a microbial consortium consist of a group of indigenous microorganisms, *A. vinelandii*, actinomycetes, bacteria and fungi petrophylic. Based on the results of [11], the application of petrophylic 2% (v/wt) shows an increase in hydrocarbon degradation rate significantly compared with the control and other treatments. And the application of mushroom log waste as a bulking agent has functioned as a porosity regulator, humidity and other sources of nutrition. Mushroom log waste has function easy to absorb and drain the water in large amounts [12].

This study focused on the assessment of the viability of *A. vinelandii*, petrophylic isolates consortium and the mushroom log waste to accelerate hydrocarbon degradation rate to rehabilitate a petroleum-contaminated soil.

2. Materials and Methods
This research was carried on in the Laboratory of Bioteknologi and Bioproses at PPPTMGB "LEMIGAS", Jakarta and Soil Biology laboratory at Agriculture Faculty of Universitas Padjadjaran. The randomized block design (RBD) was used in this experiment with six treatments and three replications consisted of: Control (A); *Azotobacter vinelandii*(B); Petrophylic (C); Mushroom log waste (D); *A. vinelandii* + mushroom log waste (E); Petrophilic + mushroom log waste (F). All of materials used in this research were the soil of the Order Ultisols from Jatinangor, oil waste was obtained from the Collection Waste PPPTMGB "LEMIGAS". Mushroom log waste was applied at 1% concentration of the soil weight. Microbial inoculant consisted of *A. vinelandii* and petrophylic with a 2% concentration of the waste load weight. Media used were PDA + 1% of the hydrocarbons, Vermani
+ 1% of the hydrocarbons, Basal glucose 2%, PDB, Actinomycetes medium, Nutrient Broth, and yeast extract.

This research was conducted in the microcosmos scale contaminated soil by 7% petroleum waste loading. The experiment stages were elaborated as follows: (1) The chemical characteristic analysis of soil and the mushroom log waste, (2) Indigenous microorganism population analysis, (2) Application of bulking agent and bioagent to soil contaminated petroleum and (3) Assessment of hydrocarbon degradation rate and chromatogram hydrocarbons analysis.

2.1. Total petroleum Hydrocarbon (TPH) analysis
The hydrocarbon degradation efficiency analysis of TPH (Total Petroleum Hydrocarbons) was conducted by reference of Test Method for Evaluating Solid Waste (US EPA Method 3540C) using Soxhlet extraction. Sample soil amount 10–25 g was mixed with 5 g of sodium sulphate or HCl in a beaker of 200 mL, and then acidified to pH 2, then added with 5 g of sodium sulfate to bind water. The mixture was put into the holster extraction, then extracted in a Soxhlet extraction set by using n-hexane. Liquid solvent extraction was done until translucent color. The TPH extracted was dried by an evaporator.

2.2. Total population of Azotobacter spp. and petrophylic bacteria analysis
Analysis of the TPC (total population counting) petrophylic bacteria was using Basal media with adding 1% of the hydrocarbons as plate media and to analysis of Azotobacter vinelandii and Azotobacter spp. indigenous were using selective media such as Vermani with adding 1% hydrocarbons. The TPC analysis was performed on each point of observation which was t0 (H0), t1 (H7), t2 (H14), t3 (H28) and t4 (H42). The features of Azotobacter vinelandii visible colonies after 24 hours of incubation were figured out by the white wet colonies will turn brown after 5–7 days of observation.

3. Results and Discussion
3.1. The hydrocarbon degradation rate
The treatment in this study consisted of six treatment variations that affected to degradation rate of petroleum hydrocarbons. The results showed that in the control treatment, whereas the degradation process took place naturally without any treatment, exhibited the degradation rate of petroleum hydrocarbons could only be achieved by 0.007811 day⁻¹, but the rate of biodegradation increased by adding A. vinelandii into 0.011038 day⁻¹ (Table 1).

| Treatments                        | Hydrocarbons Degradation Rate (day⁻¹) |
|-----------------------------------|---------------------------------------|
| Control treatment (A)             | 0.007811                              |
| A. vinelandii (B)                 | 0.011038                              |
| Petrophylic (C)                   | 0.028366                              |
| Mushroom log waste (MLW) (D)      | 0.026354                              |
| Petrophylic + MLW (E)             | 0.021285                              |
| A. vinelandii + MLW (F)           | 0.017372                              |

This figured that the performance of hydrocarbon biodegradation was triggered by the presence of A. vinelandii. This condition could be explained that A. vinelandii plays a role as a bioagent that could serve as biostimulant substances. As we know that A. vinelandii is able to produce extracellular compounds that functioned as biosurfactant [10]. The presence of the biosurfactant produced by A. vinelandii could inevitably increase the solubility of hydrocarbons in the soil system, so that the hydrocarbons which have emulsified by surfactants which were excreted by A. vinelandii and it would
be easier to degraded faster by petrophylic existing in the soil system, and also *A. vinelandii* played a role supporting N-nutrient for Petrophylic microorganisms in the soil system. *Azotobacter* spp. are the nitrogen fixer bacteria which are able to supply ammonium to the soil system and increase the N-availability. This phenomenon of *Azotobacter* spp. characteristic was also giving a positive effect on the value of the degradation rate. The treatment with *A. vinelandii* showed that the degradation rate could be increased significantly by 0.011038 day\(^{-1}\), that is much higher acceleration rate of hydrocarbon biodegradation than that of in control treatment.

The rate of biodegradation of hydrocarbons in the treatment by using MLW (D) was not significant different to the application of Petrophylic treatment (C) with the rate of 0.028366 day\(^{-1}\) and 0.028366 day\(^{-1}\), respectively. This indicated that the organic material of MLW can effectively improve the process of hydrocarbon degradation in the system without augmented petrophylic. This phenomenon happened because the MLW could force the indigenous Petrophylic performance to degrade the hydrocarbons in that system. An organic material plays a role as a bulking agent in the process of degradation of hydrocarbons and bulking agents was functioned to improve soil aeration and as a source of Nitrogen and Phosphate as well as minerals required by Petrophylic during hydrocarbon degradation underway [8]. Similarly, the mushroom waste log is also an organic material that is rich in nutrients, especially protein as a source of Nitrogen. MLW material also contain many resting hyphae of mushroom as a biomass which was rich a nutrient N and P. The existing hyphae resting of mushrooms logs waste was as a source of N and P which were easily degraded and exploited by indigenous petrophylic during the process of their metabolism. Therefore, the potential of MLW as a bulking agent to stimulate hydrocarbon degradation rate cannot be ignored. It was appearing come out that the effectiveness between MLW and petrophylic microorganism performance were similar to degrade petroleum hydrocarbon performance.

3.2. The efficiency of hydrocarbon petroleum degradation

The efficiency pattern demonstrated similar pattern of degradation rate, which was the highest efficiency achieved in the treatment petrophylic and lowest in the control treatment (Table 2).

| Treatments                          | Hydrocarbons Degradation Efficiency (%) |
|-------------------------------------|-----------------------------------------|
| Controltreatment (A)                | 39.565 a                                 |
| *A. vinelandii* (B)                 | 60.092b                                 |
| Petrophylic (C)                     | 83.940e                                 |
| Mushroom log waste (MLW) (D)        | 81.716 d                                |
| Petrophylic + MLW (E)               | 81.221 d                                |
| *A. vinelandii* + MLW (F)           | 74.788 c                                |

*A. vinelandii* increased significantly the efficiency of biodegradation compared with control treatment. Meanwhile, the degradation efficiency through the Mushroom log waste treatment was not significantly different with MLW combined petrophylic treatment. This result indicated that the material organic derived from the waste of mushroom log had a high potential in stimulating the process of rehabilitation of hydrocarbon contaminated soil and could optimally increase the efficiency of degradation processes. From that phenomenon, it can be argued that the MLW is an organic material which can play an important role in the formation of aggregates, improve soil aeration, supply nutrients and other minerals that could help increase the effectiveness of degradation of petroleum hydrocarbons process. Several microorganisms can degrade a contaminant in varying type and by adding a variety of organic matter such as straw, compost and manure that enhance PAHs biodegradation rate [13]. An organic matter can improve soil texture, transferring oxygen, and providing or supplying energy to the population degrader microorganisms in the soil system.
The growth rate of Petrophylic increased up to 0.348641 day\(^{-1}\) by addition of *A. vinelandii*. Likewise petrophylic growth rate was higher in the treatment with the addition of waste mushroom log compared to the control, that was 0.357115 day\(^{-1}\) (Table 3).

Table 3. Effect of treatments to the growth rate of petrophylic bacteria and *A. vinelandii* and its correlation to the hydrocarbon degradation efficiency for 42 days incubation.

| Treatments                      | Rate of Growth Petrophylic bacteria (day\(^{-1}\)) | Rate of Growth Azotobacter spp. (day\(^{-1}\)) |
|---------------------------------|--------------------------------------------------|-----------------------------------------------|
| Control treatment (A)           | 0.263029                                         | 0.057886                                      |
| *A. vinelandii* (B)             | 0.348641                                         | 0.086756                                      |
| Petrophylic (C)                 | 0.302718                                         | 0.219189                                      |
| Mushroom log waste (MLW) (D)    | **0.357115**                                     | **0.250719**                                  |
| Petrophylic + MLW (E)           | 0.0401639                                        | 0.083901                                      |
| *A. vinelandii* + MLW (F)       | 0.2848070                                        | 0.103146                                      |

Organic material plays an important role to create suitable conditions for improving the activity of indigenous bacteria. This role is more significant in introducing new inoculum[14]. Results showed that the mushroom log waste was important in providing optimum conditions for petrophylic in the system. This results suggested that waste of mushroom log (MLW) played an important role in providing nutrients N, P and K. This results were supported by the results of the chemical analysis of mushroom waste. This waste exhibited high organic matter content and high P potential with the ratio C:N:P of 107:2:1. This condition was expected to provide better conditions compared to control. The pH of Mushroom log waste was alkaline. This alkaline pH will help to neutralize acidic conditions when the metabolisms activity of microorganisms took place.

The potential of MLW also give a contribute to create better conditions for the growth of *Azotobacter* spp. and Petrophylic bacteria. The growth rate of *Azotobacter* spp. and Petrophylic bacteria were 0.250719 day\(^{-1}\) and 0.357115 day\(^{-1}\), respectively. The growth rate of *Azotobacter* spp. Increased five times higher than the growth rate of *Azotobacter* spp. in the control treatment. The potential of mushroom log waste as an organic material source creates an optimal condition for petrophylic and *Azotobacter* spp. population. In addition, there was an interesting finding of the experimental result that showed MLW tended to be more compatible for supporting Petrophylic indigenous. This phenomenon was proven by the treatment of a mixture of *A. vinelandii* and MLW, or MLW and Petrophylic mixture. The mixture treatments tended slower on their growth rate of *Azotobacter* spp.

The pattern of population growth and density of *Azotobacter* spp. in the soil system during the process of elimination of hydrocarbons depicts in figure 1.
Figure 1. The growth pattern of *Azotobacter* spp. at each treatment.

The treatment of MLW showed that the population of *Azotobacter* spp. increased at the 2nd week (day 14) of incubation and decreased at the 3rd week of incubation, and rised back at the 4th week of incubation. The densities of *Azotobacter* spp. until the final stages of biodegradation process were likely decline even still beyond the initial densities. While the combination treatment between *A. vinelandii* and MLW showed that *Azotobacter* spp. population density increased sharply and was relatively stable without sharp decline. This results indicated that MLW as an organic material has potential to improve and maintain existing the beneficial bacteria as *Azotobacter* spp. in the soil system. The high abundant of *Azotobacter* spp. in the soil indicated that the soil is in relatively healthy condition. Thus, MLW contributes to improving the rehabilitation process of petroleum hydrocarbon contaminated soil become healthier soil for farming land uses.

4. Conclusion
The mushroom log waste (MLW) is an organic material that potentially plays a role to accelerate the rate of biodegradation of petroleum waste and improve the efficiency of hydrocarbon degradation process. So the waste of mushroom log has a high potential as a stimulant agent for applying to the bioremediation system to rehabilitate a hydrocarbon petroleum contaminated soil become a healthier soil. The MLW is able to increase the population and maintain the density of *Azotobacter* spp., because it plays a role as a carbon source for supplying the C-availability in the soil system for microorganism growth. So the MLW gives potential contributionforremedying the soil system become healthier and safer for the media of crops and agricultural purposes.

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