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Utilization of *Talaromyces* sp., *Cladosporium* sp. and Albizia (*Paraserianthes falcataria* L. Nielsen) Mycorrhizae on the Phytoremediation of Oil Sludge: Changes of Lead, Nickel, Total Petroleum Hydrocarbon (TPH) and Polycyclic Aromatic Hydrocarbons (PAH) Contents

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Abstract

Aim: Phytoremediation is the use of microbial and rhizosphere systems to clean up a hazardous waste making it environmentally friendly,potentially zero waste and cost effective. Study on phytoremediation of oil sludge using consortium fungi (*Talaromyces* sp., *Cladosporium* sp.) and mycorrhizae Albizia sp (sengon) was conducted. This study was aimed at evaluating the ability of consortium fungi and mycorrhizae in reducing heavy metal (Pb and Ni), Total Petroleum Hydrocarbon (TPH) and Polycyclic Aromatic Hydrocarbon (PAH) contents in contaminated soil.

Methodology and results: Consortium fungi were inoculated into a compost medium containing 35% of oil sludge and monitored for two months and followed by planting mycorrhizae of sengon in the phytoremediation process. The changes of Pb and Ni, TPH, and PAH contents as well as the number of the fungi colonies in oil sludge medium were monitored every three weeks to eighteen weeks and analyzed during the remediation process. The relationship between the levels of Pb and Ni, that of TPH and the number of consortium fungi colonies were analyzed through regression correlation Thereafter, the PAH data were analyzed descriptively.

Conclusion, significance and impact of study: The results showed that good interaction between *Talaromyces* sp, *Cladosporium* sp and microoganism rhizosphere on oily sludge phytoremediation resulted in the decrement of heavy metal content (Pb and Ni), TPH and PAH compounds. On eighteenth week of observation, reduction of Pb and Ni content in 35% oil sludge medium was approximately 71.9% and 67.9%, respectively. In every increment of 1 CFU ml^{-1}, fungi consortium will affect the reduction in TPH levels to 0.286%. After fifteen weeks TPH content decreased to the lowest (70.82%), followed by the degradation of PAH compounds n-eicosane and n-hexatriacontane with carbon chains that range from C_{20}-C_{36} to the shorter carbon chain (C_{16}-C_{32}) such as Hexadecane, 2, 6,10,14-tetramethyl, heneicosane, n-hexacosane, octadecane, 3-methyl and Dotriacontane.

Keywords: Fungal consortium; Albizia mycorrhizae; Oil sludge; PAHs; TPH

Inroduction

Wastes from petroleum processing (oil sludge) are composed of hydrocarbons and heavy metals such as lead (Pb) and nickel (Ni), which are potentially carcinogenic. In accordance with the Indonesian Government Decree No. 10/2014, oil sludge is classified as hazardous and toxic waste (B3) code B 351-3.

According to the Central Laboratory of the Geological Survey (2015), oil sludge contained high levels of Pb and Ni (13.95 and 52.00 ppm, respectively). While other heavy metals (Cd, Cr, Cu and Zn) were on safe levels. The levels of Pb and Ni exceeded the standard from the Ministry of Environment Republic of Indonesia Decree No.128/2003 concerning the allowed limit of heavy metals content for Pb and Ni, which are 5.0 and 0.4 ppm, respectively. In addition, analysis conducted at the Organic Chemistry Department of Chemistry UNPAD (2015), TPH content in oil sludge was approximately 43.64%. On the other hand, PAH compounds are detected most widely in the sludge content of, for instance, Hexacosane, Tetratriacontane and Hexatriacontane. According to the stipulation of Ministry of Environment Republic of Indonesia, when the TPH content in the soil is above 15%, it must be processed or utilized in advance. In contrast, when the TPH content in the soil shows less or equal to 1%, it can be removed from the processing location and declared as safe for the environment.

Managing petroleum waste by using bioremediation will create inexpensive oil waste treatment. This technique is considered as an effective, environmentally friendly method that can produce a stable and non-toxic final compound. Utilization of microorganisms such as fungi can be applied in bioremediation due to its high ability to bind metal. One of the fungi known as detoxifiers and heavy metals absorber, such as Co^{2+}, Pb^{2+}, Cu^{2+} and Cd^{2+} is derived from the genus of *Talaromyces* and Cladospor [1,2]. A previous study demonstrated that single fungi (*Cladosporium*) is able to reduce the TPH level up to 6.67% in 35% oil sludge medium during a 60-day remediation process [3]. Single microorganism can only metabolize hydrocarbon substrate in a

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limited area. Thus, the consortium consisting a variety of fungi species with different enzymatic composition as a whole is required to increase the rate of petroleum biodegradation.

Green plants can also be used as bioremediation tools to reduce environmental pollutants, called phytoremediation. Sengon of the Albizia (*Paraserianthes falcataria* L. Nielsen) becomes one alternative tree that can be cultivated extensively for the rehabilitative purpose of the marginal lands [3]. Moreover, this plant can also associate well with mycorrhizae. Mycorrhizae are able to protect the host plants from toxic substances by filtration and accumulation on the fungal hyphae in order to prevent the entry of pollutants into the host plant cells. The objective of the present study is to evaluate the ability of consortium fungi and mycorrhizae in reducing heavy metals (Pb and Ni), Total Petroleum Hydrocarbon (TPH) and Polycyclic Aromatic Hydrocarbon (PAH) contents in oil contaminated soil.

Materials and Methods

This research employed an experimental method by providing additional treatment to fungal inoculum (*Talaromyces* sp. and *Cladosporium* sp.) into a medium containing 35% of oil sludge compost and planting mycorrhizae sengon in the phytoremediation process. The main parameters analyzed were the heavy metal (Pb and Ni), TPH and PAH contents as well as the number of fungal colonies (CFU / ml), which were analyzed every three-week intervals during the eighteenweeks of observation. The levels of heavy metals (Pb and Ni) were analyzed by using AAS (Atomic Absorption Spechtrophotometer) while TPH contents were measured using soxhlet. The number of fungal colonies (CFU ml^{-1}) was calculated using the Total Plate Count (TPC) method. The relationship between the heavy metals (Pb and Ni), TPH and fungal colonies were analyzed using regression analysis. In this study, PAH levels were measured using GCMS. In addition, supporting parameters were measured, namely the percentage of mycorrhizal infection (%) at the root of sengon using a modified staining method form Vierheilig, acidity (pH), temperature (°C), media temperature (°C) and the humidity of the media (%). Results of PAH and supporting parameters obtained were then analyzed descriptively (Figure 1).

Results and Discussion

Relationship of heavy metal content and total fungal colonies

In the present study, linear regression between the number of consortium fungi colonies (*Cladosporium* sp. and *Talaromyces* sp.) and the changes in metal content during the phytoremediation until the eighteenth week was performed (Table 1) to identify the relationship between these two parameters.

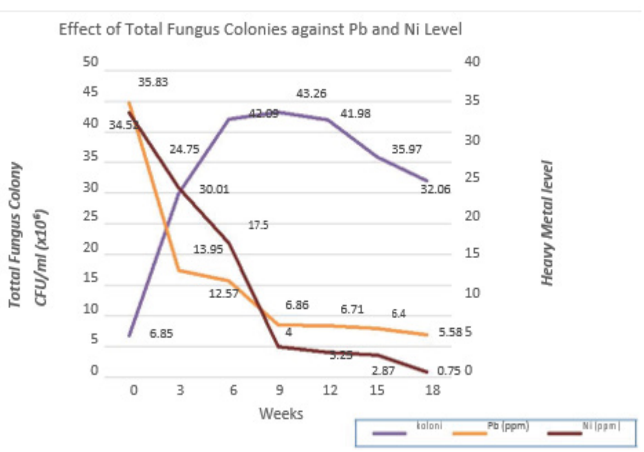

Figure 1: The effect of total fungal colonies on Pb and Ni content during 18 weeks of experiment.

The linear regression analysis indicates that there was a negative relation between the number of fungal colonies and heavy metal content (Pb and Ni). The regression equation y=13.269–0.171x showed that each additional 1 CFU ml^{-1} fungal colonies will reduce Pb content up to 0.171 ppm whereas the equation of y=39.505–0.845x demonstrates that the addition 1 CFU ml^{-1} fungal colonies will decrease Ni to 0.845 ppm. Correlation coefficient values were obtained for Pb and Ni (0.848 and 0.824,respectively), indicating a strong correlation between the number of consortium of fungal colonies and reduction in heavy metals content for both Pb and Ni. The coefficient of determination (R) for Pb was 0.719 suggesting that the reduction in Pb content was 71.9% mainly explained by the proportion of fungal colonies while the remaining 28.1% were caused by other factors. On the other hand, R value for Ni was 0.679, indicating that 67.9% of decreased Ni was strongly indicated by the number of fungal colonies . Additionally, the confidence level of regression equation for both Pb and Ni were 98.4 and 97.7%, respectively.

Effects of the total fungal colonies on Pb and Ni content

In this study, the total number of fungal consortium was calculated by using the TPC method. This method allowed the calculation of the population of fungal consortium (*Cladosporium* sp. and *Talaromyces* sp.) during the composting and phytoremediation processes. Then, the total number of fungal colonies was compared to the reduction in Pb and Ni content, up to the eighteeth week of experiment.

The number of fungal colonies was exponentially increased at the sixth week of phytoremediation experiment from 6.86×10^6 to 42.09×10^6. growth rate occurred in the ninth week of the experiment with the maximum total fungal colonies of 43.26 CFU ml^{-1}. A significant increase in the number of fungal colonies was followed by the decrease in both Pb and Ni contents (6.86 and 4.00 ppm, respectively). The reduction on both heavy metals in 35% oil sludge medium were due to metal accumulation in the medium and the degradation of polyhydrocarbon, due to the presence of fungal consortium, immobilization of heavy metals by intracellular-molecular mechanism (phytochelatins and metallothionein) and immobilization by extracellular molecules (organic acids) produced by fungi [4]. According to Sayer and Gadd, one of the metal binders that produced by fungi able to bind metal is oxalic acid. Oxalic acid is produced by microbes and may enhance microbial resistance to metals through the complex formation of metal-oxalate that are insoluble. The decline of Pb and Ni content from their initial level (84.42 and 97.82%, respectively) was followed by the fungi population of 32.06×10^6 CFU ml^{-1}.

TPH content related to the amount of fungal colonies

In the present study, a linear regression analysis between the number of fungal colonies (*Cladosporium* sp. and *Talaromyces* sp.) and TPH content during the phytoremediation process was performed (Table 2). Based on this regression analysis, the number of fungal

Variables	Pb	Ni
Regression equation	y = 13.269 – 0.171x	y = 39.505 – 0.845x
Correlation coefficient ®	0.848	0.824
coefficient of Determination ®	0.719	0.679
p- value	0.016	0.023

Table 1: Regression analysis between total fungal colonies and heavy metals contents.

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colonies negatively correlated with TPH content (regression equation y=17.8 - 0.286x). This implies that every addition of 1 CFU ml^{-1} of fungal consortium will reduce TPH content by 28.6%.

The constant value of 17.8 shows that the magnitude of TPH reduction did not affect by the number of fungal colonies consortium. In other words, the number of fungal colonies consortium were able to decrease TSH levels up to 17.8%. The Additionally, correlation coefficient values (r=0.842) indicate a strong correlation between the number of fungal colonies and the decrement in TPH content. While the correlation coefficient values (R=0.709) shows that 70.9% reduction in TPH is mainly explained by the ability of consortium fungal colony, while 29.1% was influenced by other factors.

Effect of total fungal colonies against TPH levels

In this study, the fungal population during thecomposting and phytoremediation processes were compared to the degradation of TPH until the end of experiment (18th week).

Total fungus and TPH levels graph

The number of fungal consortium exponentially increased in the third week of the composting process, i.e., from 6.85 × 10^6 to 1.30 × 10^6 CFU ml^{-1}. This increasing number of colonies was followed by 31.15% reduction of TPH content (Figure 2). After six weeks of composting, the phytoremediation process was started by planting Mycorrhiza sengon as a media into the composting for 12 weeks. The fungal consortium (*Cladosporium* sp. and *Talaromyces* sp.) reached their optimum growth on the ninth week of the experiment (43.26 × 10^6 CFU ml^{-1}) and successfully reduced the TPH up to 58.6%. In addition, the effects of temperature and humidity in biodegradation process can also affect the amount of the microbial population and also the degradation rate of hydrocarbons. Maintaining the media temperature between 25-27°C and humidity of 47.6-60.2% is considered as the optimum condition for biodegradation. High temperatures may increase the enzyme activity and the volatility of toxic short-chain alkanes. In contrast, the solubility of these alkanes in the Polycyclic Aromatic Hydrocarbon (PAH) content.

In our study, there were nine main hydrocarbon compounds detected from the beginning to the end of remediation experiment,

Regression equation	y = 17.8 – 0.286x
Correlation coefficient (r)	0.842
Coefficient of Determination (R)	0.709
p-value	0.018

Table 2: Linear regression between total number of fungal colonies and TPH content.

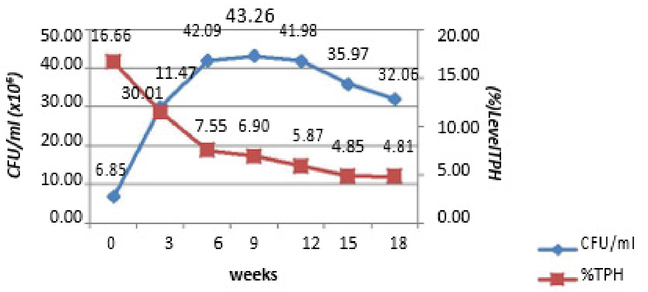

Figure 2: Total fungal colonies (CFU ml^{-1}) and TPH content (%) during phytoremediation experiment.

namely: Hexadecane, 2,6,10,14-tetramethyl, n-Eicosane, heneicosane, n-Hexacosane, octadecane, 3-methyl, Hexatriacontane, Dotriacontane, Eicosane, water will decreased, thus speeding up the rate of biodegradation [5]. Medium humidity above 70% may disrupt the transfer of oxygen, thereby reducing aerobic activity. The organic acid metabolites produced from the metabolic activity of the yeast cells during the degradation of hydrocarbons such as fatty acids would further oxidize to acetic and propionic acids. Indeed, acidity has a crucial role in biodegradation. Thus, when the soil or media has a tendency to approach the acidic condition, indicating that the composting did not perfectly mature. 2,4-dimethyl and n-Tricosane. Such compounds were categorized as C_{16} up to C_{36} of carbon chains. Change in the proportion on these compounds occurred during the remediation phase from the first to the eighteenth week. The changes of the proportion of each hydrocarbon is presented in Table 3.

The hydrocarbons were still detectable by the GC/MS during the remediation phase from the first to the twelfth week. This could be due to heavy metals contained in the media so that the treatment could hinder the activities of microbial enzymes to degrade the hydrocarbons. Afterwards, on the fifteenth week of the experiment, the compound of n-eicosane and n-hexatriacontane with C_{20} and C_{36} carbon chains degraded and became undetectable until the end of remediation (Table 3). also, the eicosane compound, 2,4-dimethyl and n-Tricosane, which had C_{20} and C_{23} carbon chains, also degraded entirely. Based on the growrh curve graph, the fungal consortium (*Cladosporium* sp. and *Talaromyces* sp.) entered the stationary phase on twelfth week of experiment. According to Thaniyavarn, small amounts of biosurfactant are produced in the early exponential phase, subsequently the production increased during the end of exponential and static phases due to the activity of the enzyme generated in optimum conditions. Additionally, the presence of microbial activity in degrading petroleum sludge performed by cutting the component of long-chain aliphatic hydrocarbons and transforming the aromatic hydrocarbon compounds. Therefore, the changes in the structure composition of hydrocarbon fraction may occur.

Generally, the changes in carbon chain and a number of compounds detected during remediation showed the breakdown of complex carbon chains into simpler carbon chains. This was demonstrated by the compounds which were still detectable at the week eighteenth of the experiment, i.e., Hexadecane, 2, 6, 10, 14-tetramethyl, heneicosane, n-hexacosane, octadecane, methyl while the chain of CH Dotriacontane showed a simpler range of carbon chain (C_{16} to C_{32}). Sharpley and Nugroho estimated that the hydrocarbon bonds.

Percentage of infection of mycorrhizae in sengon roots

The examination concerning the initial infection was carried out to determine the existence of mycorrhizal infection in sengon during

Compound name	Total C	Weeks						
		0	3	6	9	12	15	18
Hexadecane, 2,6,10,14-tetramethyl	16	√	√	√	√	√	√	√
n-eicosane	20	√	√	√	√	√	-	-
Heneicosane	21	√	√	√	√	√	√	√
n-hexacosane	26	√	√	√	√	√	√	√
Octadecane, 3-methyl	18	√	√	√	√	√	√	√
n-hexatriacontane	36	√	√	√	√	√	-	-
Dotriacontane	32	√	√	√	√	√	√	√
Eicosane, 2,4-dimethyl	20	√	√	√	√	√	√	-
n-Tricosane	23	√	√	√	√	√	√	-

Table 3: The presence of hydrocarbon compounds in the remediation medium.

phytoremediation. The result showed that the level of mycorrhizal infection in the sengon root was classified to class 3 (26-50%) with moderate infection rates (Table 4). In addition, mycorrhizae were able to adapt in 35% media oil sludge and fungal consortium (*Cladosporium* sp. and *Talaromyces* sp.). After the phytoremediation, mycorrhizal infection at the root was classified in Class 5 (76-100%) categorized as high infection rate (Table 4). The percentage of mycorrhizal infection at Mycorrhizae infection increased in heavy metals-contaminated environments, also it could act as protector to the host plants in reducing heavy metals toxicity. According to Lakitan (2001), mycorrhizae could bind with the metal ions in the cell walls of hyphae keeping it in crystalloid mycelium break heavy fractions into lighter fractions causing multiplication in this lighter hydrocarbon. the roots of sengon plants can be seen in Table 4.

Before phytoremediation

Fungi and cortical cells of Mycorrhizae plant roots. Furthermore, Khan [6] estimated that the presence of mycorrhizae could maintain the stability of plant growth in the polluted soil. Sengon is, indeed, considered as a hyper accumulator plant that has the ability to dissolve metals in the rhizosphere. The mechanism of metals hyper accumulation could occur through the rhizosphere interaction, which is the interaction between plant roots and soil media. When the plants absorb the heavy metals, the membrane in the roots will automatically form reductase enzyme, which serves to reduce the metals being transported through special mechanism in the membrane of the roots. The observation of mycorrhizal infection was conducted microscopically at 100x magnification and presented in Figure 3.

The results suggest that the phytoremediation was neither related to the decrement of TPH content nor the changes in PAH before or after mycorrhizal infection. The role of mycorrhizal infection at the sengon root was as rhizofiltration, to protect the root from the absorption of toxic compounds. Indeed, the presence of mycorrhizae in the rhizosphere was to help provide the nutrients of plants that were limited due to the presence of hydrophobic contaminants such as PAHs. These contaminants can enter through hyphae and penetrate deeply into the soil, increasing the activity of the dehydrogenase enzyme, phosphatase and nitrogenase [7].

Conclusion

Apparently, the plant roots were able to produce root exudates approximately 10% to 20% from the metabolism. The photosynthesis

Replicates	Infections percentage (%)	Infection level
1	50	III
2	49	III
3	50	III
4	48	III
5	50	III
6	50	III
After Phytoremediation		
Replicates	Infections percentage (%)	Infection level
1	100	V
2	100	V
3	100	V
4	100	V
5	100	V
6	100	V

Table 4: Percentage of mycorrhizal infection at sengon roots.

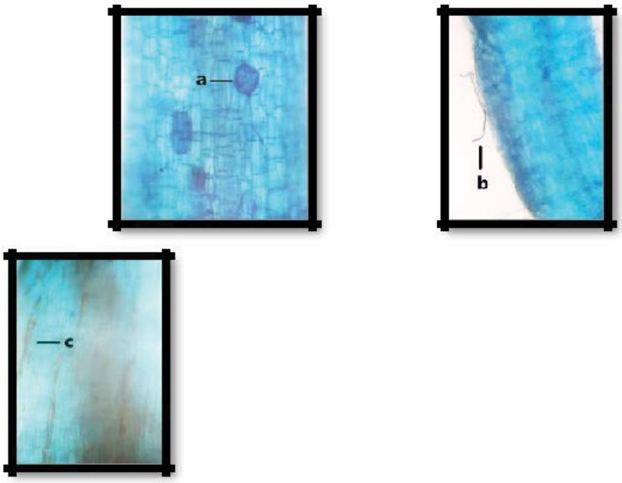

Figure 3: Mycorrhizae Infection in microscopic description: A: Vesicle, B: External hypae, C: Internal hypae. (Source: Personal Documentation, 2015).

produces sugars, amino acids and organic acids that may be able to enhance the activity of microorganisms in the roots and soils. This fact is in accordance to Brandt [8] that rhizosphere did not actually affect TPH reduction. Nevertheless, the rhizosphere constitutes an important system in phytoremediation of crude oil, since it could create favorable conditions for microbes to cause degradation of the contaminants. In the present study, we observed a negative correlation between the number of fungal consortium (*Talaromyces* sp. And *Cladosporium* sp.) and heavy metals (Pb and Ni) and TPH content during the 18 weeks of remediation process. The fungal consortium and mycorrhizae in sengon were able to degrade Pb and Ni contents up to 71.9 and 67.9% from their initial concentration. Reduction in Pb and Ni contents to the lowest level occurred on the last week of remediation while reduction in TPH went [9].

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