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Manganese (Mn) stress toward hyperaccumulators plants combination (HPC) using *Jatropha curcas* and lamtoro gung (*L. leucocephala*) in mychorrizal addition on soybean (*Glycine max*) seedling stage

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Biodegradation of Naphthalene and Phenanthren by *Bacillus subtilis* 3KP

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Abstract. The purposes of this research were to know growth response, degradation ability, and uptake mechanism of naphthalene and phenanthrene by *Bacillus subtilis* 3KP. *Bacillus subtilis* 3KP was grown on Mineral Synthetic (MS) medium with addition of 1% yeast extract and naphthalene and phenanthrene respectively 200 ppm in different cultures. *Bacillus subtilis* 3KP growth response was monitored by Total Plate Count (TPC) method, the degradation ability was monitored by UV-Vis spectrophotometer, and the uptake mechanism of hydrocarbon was monitored by emulsification activity, decrease of surface tension, and activity of Bacterial Adherence to Hydrocarbon (BATH). *Bacillus subtilis* 3KP was able to grow and show biphasic growth pattern on both of substrates. Naphthalene and phenanthrene were used as a carbon source for *Bacillus subtilis* 3KP growth that indicated by the reduction of substrate concomitant with the growth. At room temperature conditions (± 30^0C) and 90 rpm of agitation for 7 days, *Bacillus subtilis* 3KP could degrade naphthalene in the amount of 70.5% and phenanthrene in the amount of 24.8%. Based on the analysis of UV-Vis spectrophotometer, three metabolites, 1-hydroxy-2-naphthoic acid, salicylic acid, and pyrocatechol were found in both cultures. The metabolite identification became basis of propose degradation pathway of naphthalene and phenanthrene by *Bacillus subtilis* 3KP. The results of hydrocarbon uptake mechanism test show that *Bacillus subtilis* 3KP used all of the mechanism to degrade naphthalene and phenanthrene.

Keyword: *Bacillus subtilis* 3KP, Biodegradation, Biosurfactant, Phenanthrene, Naphthalene

INTRODUCTION

Naphthalene and phenanthrene are types of hydrocarbon that classified as polycyclic aromatic hydrocarbon (PAH). This kind of compound can cause damage on mammal tissue when it is exposure in high dose (Patnaik, 1994). The existence of PAH, as pollutant, is often yield from exploration and production in petroleum industry. Petroleum waste sludge (oil sludge) consists of hydrocarbon compounds such as aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAH), water, metals and non-hydrocarbon compounds such as nitrogen, sulfur, oxygen, and asphalt (Connell and Miller, 1995). PAH almost reaches amount of 13.24% in petroleum (Yuliani, 2014).

A method called bioremediation has become an important method to solve the petroleum waste by using indigenous or exogenous microbes (Helmy et al., 2010). The progress on degradation study showed how difficult to degrade PAH because of its stability and recalcitrantly in soil. Less of solubility also limits bioavailability and efficiency of bioremediation process.

In biodegradation process of PAH, it is often mentioned about the role of biosurfactant on biodegradation. Biosurfactant could increase solubility and availability of substrate for bacteria (Li, 2009). Besides

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biosurfactant, some enzymes also have been linked with biodegradation process of hydrocarbon, especially PAH. The existence of enzyme enable bacterial cell degrade hydrocarbon polymer into simpler compound. So, it can be used as carbon source. Various types of enzyme also related with hydrocarbon uptake mechanism of bacteria.

Bacillus subtilis 3KP was bacteria which known as hydrocarbon degrading bacteria. These bacteria are also able to produce biosurfactant. According to Ni'matuzahroh et al. (2013), *Bacillus subtilis* 3KP was able to produce biosurfactant in the amount of 12.3 g/L using molasses substrate. *Bacillus subtilis* 3KP was potential to degrade PAH compound, but the research has not ever done yet. This research aims to observe the bacterial growth, biodegradation percentage of PAH, and mechanism of biodegradation that was probably developed by *Bacillus subtilis* 3KP.

MATERIALS AND METHODS

Chemicals

All chemicals were analytical grade or better. Naphthalene (99%), phenanthrene (99%), 1-hydroxy-naphtoic acid (99%), salycylic acid (99%), and pyrocathecoid acid (99%) were purchased from Sigma-Aldrich. Stock solution of 1-hydroxy-naphtoic acid, salycylic acid, and pyrocathecoid acid were prepared in ethyl acetate (MERCK). Naphthalene and phenanthrene were prepared in mineral salts medium (MS, see below) and sterilized by autoclaving before the bacteria were inoculated.

Microorganism and Growth Medium

The following bacteria used are *Bacillus subtilis* 3KP which isolated from petroleum-contaminated environment in Donan River, Cilacap, Indonesia. *Bacillus subtilis* 3KP was grown in Nutrient Broth and Nutrient Agar before inoculation.

All media used in these studies were prepared in distilled water. The MS medium from the method by Pruthi and Cameotra (1997) contained (L^{-1}) 3 g $(NH_4)_2SO_4$, 0.2 $MgSO_4.7H_2O$, 10 g NaCl, 0.01 $CaCl_2$, 0.001 g $MnSO_4.H_2O$, 0.001 g H_3BO_3, 0.001 g $ZnSO_4.7H_2O$, 0.001 g $CuSO_4.5H_2O$, 0.005 g $CoCl_2.6H_2O$, 0.001 g $Na_2MoO_4.2H_2O$, and 1% yeast extract. The buffer composition contained (L^{-1}) K_2HPO_4 and KH_2PO_4 for respectively 2.6207 g and 5 g in 50 ml. It was also added 0.0006 g $FeSO_4.7H_2O$ which sterilized separately in 50 ml distilled water. The pH of the final medium was adjusted to 7.0. For preparing inoculation, bacterial cultures were suspended in 0.85% salt solution.

Determination of Residual Substrat

Biodegradation tests were performed in 250 ml flask and run in duplicate. *Bacillus subtilis* 3KP (5%) (v/v) was grown in 30 ml MS medium. Naphthalene and phenanthrene were added to the medium 200 ppm respectively. The flasks were shaken on a rotary shaker with 90 rpm at 30°C for 7 days. All cultures were started with cell density $OD_{610\ nm}$ of 0.5. Growth profile was observed by common serial dilution. The residual hydrocarbons and the bacterial growth were investigated at days 0, 1, 3, 5, and 7.

At the same time of interval, analysis of hydrocarbon residue in culture was observed by UV-vis spectrophotometer (Kumar et al., 2010). Before analysis, the culture was extracted by adding ethyl acetate to the separating funnel in ratio 1:1. Then it was shaken twice for 15 minute in each shaking. Separating of liquid phase, emulsion, and solvent phase was done by adding absolute ethanol. The data was shown as absorbance value at optimal wavelength 276 nm for naphthalene, 293 nm for phenanthrene, 339 nm for 1-hydroxy-2-naphthoic acid, 304 nm for salicylic acid, and 278 nm for pyrocatechol. Standard curve was used to determine concentration of naphthalene or phenanthrene in the culture. Hydrocarbons biodegradation percentage was calculated with the formula:

$$Biodegradation\ (\%) = \left(\frac{initial\ concentration\ of\ hydrocarbon - final\ concentration\ of\ hydrocarbon}{initial\ concentration\ of\ hydrocarbon} \right) x\ 100\%$$

Cell surface Hydrophobicity

According to Rosenberg et al. (1980), the percentage of the cells adhering to an oily phase was investigated by using a modification method. The bacterial cells were harvested each 48 hours by centrifugation (3000 rpm, 15 minute). The cell pellets were washed twice and suspended in phosphate buffer (g L^{-1}; 7.3 KH_2PO_4 and 16.9 K_2HPO_4), then diluted to an optical density (OD) of 0.5 at 610 nm. Into the cell suspension (4 mL) in test tube,

100 µL of hexadecane was added and vortex-shaken for 3 minute. After forming the suspension, it was allowed to separate for 60 minute, the absorbance OD_{610} in the aqueous phase was measured directly. The clearing measurement of the aqueous phase indicated the capacity of adherence cell to the oily phase. The result is presented as percentage with formula:

$$H\ (\%) = \left(1 - \frac{A}{A0}\right)\ x\ 100\%$$

H was hidrophobicity; A was cell suspension absorbance after added by hexadecane; A0 was initial cell suspension absorbance.

Surface Tension Reduction

All experiments were performed using two separately grown bacterial cultures. The cultures were centrifuged at 3000 rpm for 15 minute. Then, the surface tension of supernatant was measured by Du-Nouy tensiometer. The result is presented as mN m^{-1} and reported as an average of triplicate each sample.

Emulsification Activity Assay

Emulsification activity was measured using the method by Cooper and Goldenberg (1987). The supernatant (1 mL) as yield of centrifugation was added to a test tube containing 1 ml kerosene. The mixture was homogenized by vortexing for 2 minute and the emulsion was allowed to stand for 60 minute to be measured. Emulsification index was determined as ratio of the emulsion height to total height of the mixture, then it was calculated their percentage.

RESULTS AND DISCUSSIONS

Biodegradation process of PAH is affected by several factors, those are microbial character, environmental factor that support the growth, and characteristic of substrate. Microbial ability in degradation of hydrocarbons depends on its adaptation, toxic resistance, and gene expression of the microbe (Abbasnezhad et al., 2011). Microbial ability in degrade hydrocarbon could be known through the growth, the use of hydrocarbon as sole carbon, and the secondary metabolites that produced by microbe.

Bacillus subtilis 3KP grew up and showed positive responses to naphthalene and phenanthrene, 200 ppm respectively. *Bacillus subtilis* 3KP was able to adapt and use PAH as a sole carbon (Fig.1). This is proved by decreasing of concentration of substrate accompanied by increasing of the cell amount. The growth pattern of *Bacillus subtilis* 3KP on naphthalene and phenanthrene showed a biphasic pattern. In compare to control study that showed a normal phase, this kind of pattern indicated that *Bacillus subtilis* 3KP has adapted to the content of PAH in the medium.

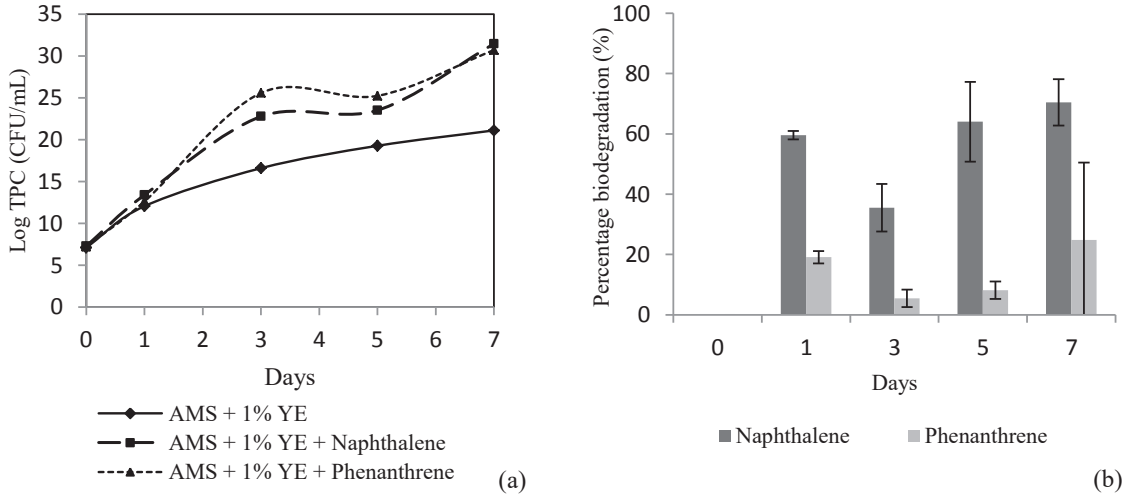

FIGURE 1. (a) *Bacillus subtilis* 3KP growth response in the naphthalene and phenanthrene substrates, (b) Percentage of naphthalene and phenanthrene biodegradation at several days

Stationary phase occurs when cell growth was inhibited by cell density, accumulation of secondary metabolites that could be toxic and limited oxygen (Pelczar, 1986). In this study, accumulation of intermediate compound was found in the end of exponential phase, those are 1-*hydroxy*-2-*naphthoic acid* and *salicylic acid.* According to metabolic pathway of phenanthrene degradation by bacteria, 1-*hydroxy*-2-*napthoic acid* would be transformed to 1-2-*dihydroxynaphthalene* before it became *salicylic acid.* Pumphrey and Madsen (2007) have explained that 1-2-*dihydroxynaphthalene* could be oxidized to a toxic 1-2-*naphthaquinon* which inhibited the growth of *Polaromonas naphthalenivorans*. The intermediate compounds of naphthalene and phenanthrene metabolism can be accumulated in culture because of their easy-soluble character and more toxic while they have increased continuously.

Some microorganisms are able to hold out from naphthalene and phenanthrene and to transform them to enter the TCA (*tricarboxylic acid*) cycle, *Bacillus subtilis* 3KP did it. At day 7, *Bacillus subtilis* 3KP has degraded 70.5% naphthalene and 24.8% phenanthrene. Intermediate compounds have been observed in the study, those are 1-*hydroxy*-2-*napthoic acid* and *salicylic acid*. *Pyrocatechol* was used along with the use of main substrate. It showed that *Bacillus subtilis* 3KP has degraded naphthalene and phenanthrene in a same metabolic pathway. Data existence of intermediate was shown in table 1.

TABLE 1. The existence of intermediate compounds during the process of degradation of naphthalene and phenanthrene by *Bacillus subtilis* 3KP

Days	Existence of intermediate compounds					
	1-*hydroxy*-2-*napthoicacid*		*Salicylic acid*		*Pyrocatechol*	
	Naphthalene	Phenanthrene	Naphthalene	Phenanthrene	Naphthalene	Phenanthrene
0	+	-	+	-	+	+
1	+	-	+	-	+	+
3	+	-	+	-	+	+
5	+	-	+	-	+	+
7	+	-	+	+	+	+

Biodegradation process of naphthalene and phenanthrene depends on the ability of microorganism to produce enzymes. It was assumed that there was an important role of *dioxygenase, hydrogenase,* and *monooxygenase* in biodegradation process of naphthalene and phenanthrene by *Bacillus subtilis* 3KP. Enzyme regulation in naphthalene and phenanthrene is affected by existence of gen. They were nah and phn which take the role in biodegradation of naphthalene and phenanthrene (Laurie and Jones, 1999). For this research, the existence of catabolic gen in *Bacillus subtilis* 3KP should be investigated as advanced research to affirm its ability of PAH degradation.

Moreover, hydrocarbon properties also affect biodegradation process. Complexity of structure, molecular weight, and solubility of the hydrocarbon become a limiting factor microbe's access to hydrocarbons. *Bacillus subtilis* 3KP was released biosurfactant to contact with the hydrocarbons. There was a similar growth pattern and uptake mechanism of substrate by *Bacillus subtilis* 3KP that indicated in Figure 2.

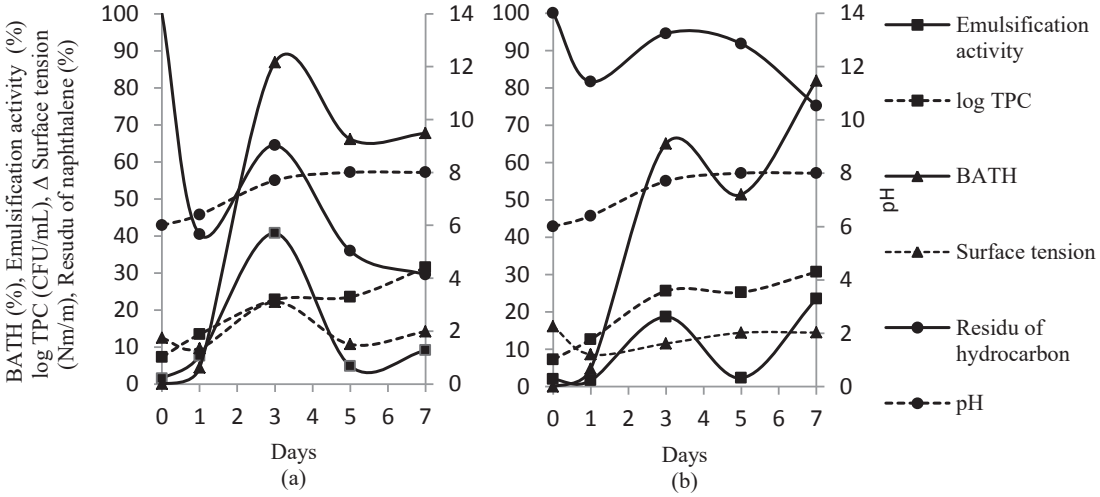

FIGURE 2. Mechanism of naphthalene (a) and phenantrene (b) biodegradation by *Bacillus subtilis* 3KP

At the early stationary phase, emulsification activity and adherence activity increased, while surface tension reduced. Extracellular biosurfactant of *Bacillus subtilis* 3KP is able to reduce the surface tension of culture supernatant at the early stationary phase. It was in accordance to Cooper and Goldenberg (1987), the lysis of *Bacillus cereus* in *sucrose* was increasing the biosurfactant activity as a *surface active agent* because of its polysaccharide (emulsifier) and lipid reduced the surface tension. So that, at the early of stationary phase, emulsification activity increased together with the reducing of surface tension. It was assumed that *Bacillus subtilis* 3KP was secreted polypeptide-type biosurfactant.

Biosurfactant consists of surfactant monomer that would form stable micelle from about 10 – 200 molecules (Makkar and Rockne, 2003). Scippers et al. (2000) explained about the role of biosurfactant in biodegradation of PAH. The first role, micelle was used as a PAH porter to microbial cell wall surface, then PAH was entered diffusely the cell. The second role, micelle could increase PAH solubility in the medium. The last role, without micelle, surfactant still could facilitate the use of PAH by a cell-surfactant-PAH contact directly (bacterial adherence to hydrocarbons).

In pH 6 – 8, *Bacillus subtilis* 3KP are able to grow. Emulsification activity and surface tension reduction were not affected by increasing in pH. Therefore, at day 7 when the value of pH showed 8.0, emulsification activity was still rising. While in surface tension, there was not a significant difference.

Biosurfactant takes role in biodegradation process in accessing not soluble substrate. It was showed from the result that emulsification activity and surface tension reduction in culture with naphthalene was higher than with phenanthrene. However, the adherence activity was decreased after the end of exponential for culture with naphthalene. While in culture with phenanthrene, adherence activity has increased much higher at day 7. It was assumed that in culture with more complex structure, cells were adapted to transform its cell wall to be more hydrophobic. Prabhu and Phale (2003) have learned about uptake mechanism of aliphatic and aromatic hydrocarbons by *Pseudomonas* sp. strain PP2. Cell hydrophobicity raised after the decreased of emulsification activity in stationary phase. Level of cell hydrophobicity was higher in aromatic which is a lower soluble compound than aliphatic one.

At the same relatively environmental conditions and with the same type of microbe, phenanthrene degradation was longer than the naphthalene degradation. It proves that the type of substrate affects the biodegradation process. Although naphthalene and phenanthrene are classified as a some group of polycyclic aromatic hydrocarbons, naphthalene has much higher solubility than phenanthrene. Low substrate solubility in the medium can inhibit the bacterial access to the substrate. Limited of available substrate is the most important factor that involved in the slow degradation of PAHs (Makkar and Rockne, 2003).

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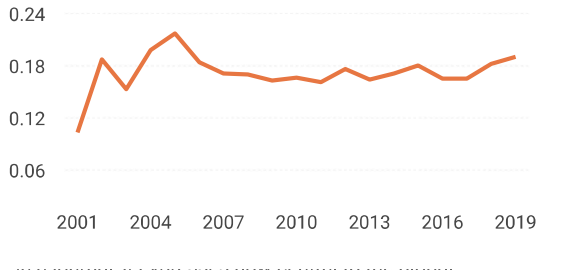

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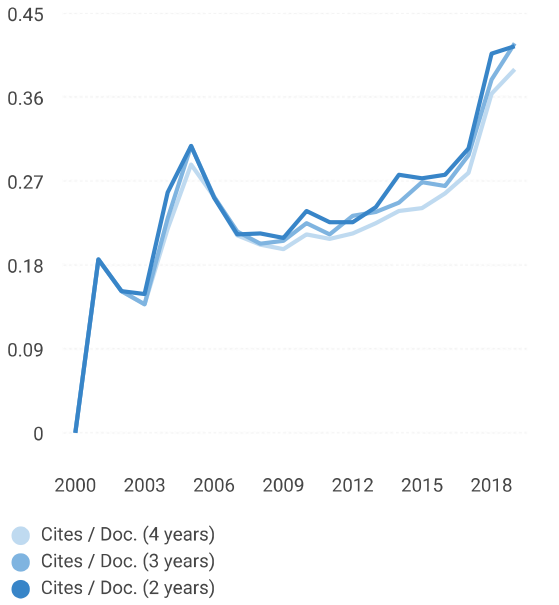

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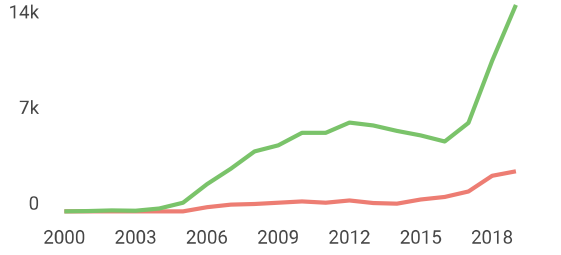

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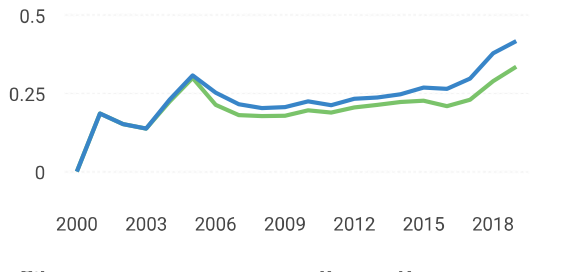

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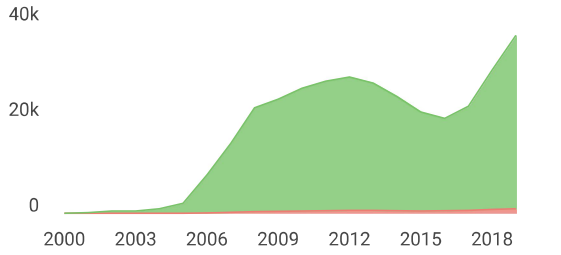

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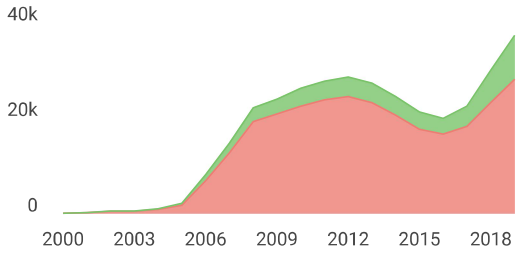

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