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Biotransformation of Heptachlor to Hydroxychlordene by Soil Bacteria.

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ABSTRACT

Heptachlor is an organochlorine pesticide that can persist in the environment, resulting in environmental problem with chronic effects on human and animal health. The determination of heptachlor in soil samples from 9 agricultural areas in Karawang district, West-Java, Indonesia central of paddy field, found heptachlor residue in the ranges of 0.3-4.5 ng/g soil. From representative of these sampled areas and after repeated culturing in nutrient agar 5 morphologically different bacterial strains were isolated. Out of isolates, bacteria potentials was showed heptachlor degrading ability as indicated by protein increase when grown on Medium Mineral (MM) supplemented up to 0.2-2.0 µg/g. Growth of these isolates in MM+heptachlor 2.0 µg/g after 30 days indicated reductions of heptachlor until 3.99 ng/g. Based on GC analysis, showed the new peak in time (rt) 10.78 minute and consistence all of concentration was 1-hydroxychlordene. And, the morphological characteristics and 16S rDNA analysis, isolate B4 which showed the highest degradation ability was found to be 99% identical (1360/1362) to *Raoultella ornithinolytica* B4.
Keywords: heptachlor, biotransformation, soil bacterium

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INTRODUCTION

Heptachlor, a chlorinated dicyclopentadiene insecticide targeted by the Stockholm Convention on Persistent Organic Pollutans, persist long in the environment and accumulates in the food chain. Organochlorine was used for soil insect pests and was used extensively on farmland until 1988 in Indonesia. In approximately 26 years since the ban on the use of heptachlor in Indonesia farmland, this compound are still detected in environmental compartements (paddy soil, water, sediment, and agriculture product) [1].

The biodegradation of persistens compounds is an important mechanism for their dissemination in the environment [4]. Although a large amount of heptachlor had been used as a pesticide in field soils, there is little microorganisms play an important role in organochlorine pesticides degradation. Microorganisms play an important role in the convertion of cyclodiene insecticides in soil to nontoxic product. . Microbial degradations of organochlorine pesticides have been observed under both aerobic and anaerobic conditions. Aerobic degradation of heptachlor by several type of bacteria have been reported such as *Bacillus subtilis* RS-01, *B. cereus* RS-02 and *Pseudomonas putida* RS-03 were dominant in all biotic studies [3]. Pure culture of a range of soil microorganisms have been reported to transform endosulfan to a nontoxic diol metabolite in unsealed liquid culture by 28 soil fungi, 14 soil bacteria, and 10 soil actinomycetes [4].

The specific objectives of the study are isolation of natural microorganism that capable of degradating heptachlor, and evaluation of their degradative capability under condition of carbon free media (medium mineral (MM)).

MATERIALS AND METHODS

Equipment and Material

Field Gas Chromatography (Varian), ECD (63Ni), Gas Chromatography-Mass Spectra (Agilent Technology Type 7890 A-Type 5975C), Rotary evaporator, Spectrophotometer (Genesis 20), Laminar Air Flow, Colony-counter, hand counter, and glassware. Materilas used top soil surface (1-100 cm) from 9 sites from paddy area in Karawang district, West-Java, Indonesia. K_2HPO_4, $CaCO_3$, $FeSO_4.7H_2O$, NaCl, and $MgSO_4.7H_2O$, $MnSO_4.4H_2O$.

Methods

Soil Samples

Areas of Samples: top soil surface (0-100 cm) samples were collected from nine sites from agricultural area in Karawang District, West-Java, Indonesia and used in this study for isolation of heptachlor degrading microorganisms.

Sample Collection Methods: A soil auger of 20 cm length was used randomly collect the soil samples were taken from different location and mixed thoroughly to make composite sample. The collected samples were placed in paper bags, labeled and immediately transported to the Residual Pesticide Laboratory, Bogor.

Preparation of Samples: The samples were left over night to dry in open air or at room temperature. Each sample was then mixed thoroughly.

Preparation of Mineral Medium: One liter of liquid media was prepared to one conical flasks. One g K_2HPO_4, 0.05 g $CaCO_3$, 0.001 g $FeSO_4.7H_2O$, 0.5 g NaCl, and 0.5 g $MgSO_4.7H_2O$, 0.01 $MnSO_4.4H_2O$ (modified from [4]). The volume was completed to one liter distilled water. The flasks containing these media were autoclaved for 15 minutes at 121 ^oC, allowed to cool at room temperature and kept in the refrigator as stock media at 5 ^oC.

Degradation of Heptachlor in Mineral Medium: A total of 10 conical flasks (100 mL) and culture media (mineral medium) were autoclaved separately for 15 min, at 121 ^oC. Fifty mL of culture media was added inoculums and one mL of 0.2-2.0 µg/g heptachlor to each flask. All flasks were incubated at 29-30 ^oC for 30 days. Certain amount of sample were collected every 7 days for the determination of heptachlor concentration and turbidity, and cell growth via total protein measurement by Lowry's method.

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Extraction and Analysis: Approximatelyt 10 mL samples were taken from each flask every five days, extracted by aceton solvent and analyzed used GC Trace 1300, Detector ECD, Colum Rx-5. Brief condition Oven temperature 350 ^{o}C, Injector: 250 ^{o}C, Spin Flow 50 mL/min.

RESULTS AND DISCUSSIONS

Quantitative determination of pesticides and bacterium in soil sample

Soil samples from total of 9 sites in Karawang district areas were collected, of which 8 were analyzed and found to be contaminated with heptachlor in range 0.3-4.5 ng/g soil (Table 1). Karawang Timur district none heptachlor than the other sites. The areas under investigation have a history of pesticides usage for more than 30 years and, although, organochlorine has been banned since the 1980s it still being used as the soil inseciside. Heptachlor generally persist in the environment depending on soil and compound compositions. In general, the amount of pesticides adsorbed on soils has been reported to related to the organic carbon contens of soils [5].

The bacterial consortium isolated from sample was made up of a group of strains whose action was reflected in significant insecticide depletion (Table 2). Unfortunely laboratory methods are only capable of isolating 1-10% of all bacteria growing in soil, so several of the bacteria interfering in the degradation processes in natural environments cannot be obtained in a laboratory. From the results of this study, we cannot be sure whether this hydrolysis subsequently results in complete mineralization or if the resulting metabolite in the medium. Proof of this the isolation of bacteria from paddy soils where repeated aplications of OP pesticides have been reported [6].

Table 1: Amount of heptachlor residue in The District Karawang (Desember 2013 and Desember 2014).

No	Subdistrict	Heptachlor (ng/kg)	
		2013	2014
1.	Rengasdengklok	4.3	-
2.	Batujaya	1,2	-
3.	Tirtajaya	0.6	-
4.	Pedes	4.5	4.4
5.	Cilebar	0.3	-
6.	Tempuran	1.9	6.6
7.	Cilamaya Wetan	0.9	3.7
8.	Jatisari	3.7	5.8
9.	Karawang Timur	-	10.9

Table 2: Total amount of soil bacteria from soils sample.

No	Depth (cm)	Bacterial	
		Genus	Σ (CFU/g)
1.	0-25	**Citrobacter**	**1.32×10^{8}**
		Pseudomonas	8.0×10^{7}
		Bacillus	8.4×10^{7}
		Enterobacter	2.5×10^{7}
2.	50-75	**Citrobacter**	**2.3×10^{6}**
		Pseudomonas	1.6×10^{8}
		Bacillus	2.68×10^{8}
		Azotobacter	1.4×10^{5}
		Azospirillium	1.9×10^{5}
3.	75-100	**Citrobacter**	**7.8×10^{7}**
		Pseudomonas	9.0×10^{5}
		Bacillus	1.2×10^{6}
		Azotobacter	3.1×10^{5}
		Acetobacter	2.4×10^{8}
		Sphaerotilus	8.0×10^{5}
		Flavobacterium	2.4×10^{7}

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Biotranformation of Heptachlor in Medium Mineral by bacterial isolates

The bacterial consortium isolated from the sample was made up of a group of strains whose action was reflected in significant pesticide depletion. Microbial degradation is the breakdown of chemicals by microorganism. It occurs when fungi, bacteria, and other soil microorganisms use pesticides as food or consume pesticide along with other substances. Microbial degradation occurs at a higher rate in the surface soil horizons, particularly on areas with high organic matter.

The bacterial population in soil samples collected for screening was in the ranges 1.4 x10^5- 2.4 x 10^8 CFU/g soil (Table 2), selected bacterial isolates were found to grow and degrade heptachlor within 30 days. Each of bacteria, *Citrobacter* sp isolate was inoculated into MM+heptachlor medium, after which growth profile and heptachlor level were followed during the 30 days of cultivation. The results indicate that 100% of heptachlor was removed from MM (Figure 1). Additionally, figure 2 show growth and protein showed *Citrobacter* sp still grow better in MM with an addition of heptachlor 0.2-2.0 µg/g. It can be observed that the concentration of heptachlor decreased by almost 100% in the culture during 30 day cultivation. Under these conditions, the bacteria Citrobacter sp could be effective for treating wastes or restoring contaminated environments. Results indicated that these isolate could used heptachlor as sole carbon and energy sources.

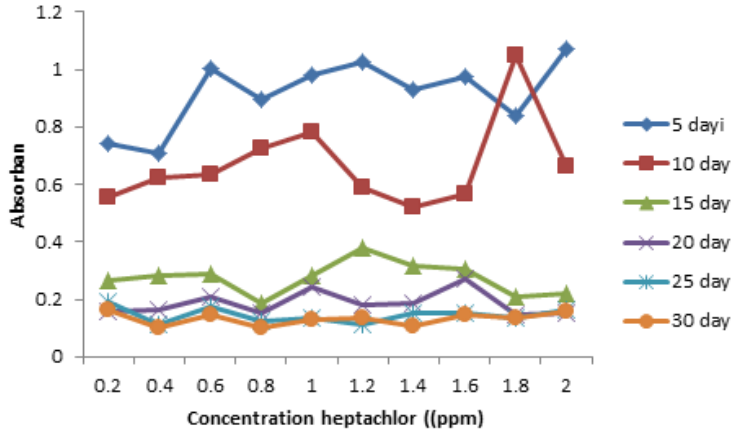

Figure 1: Optical Density (OD) of bacteria until 30 days incubation in heptachlor

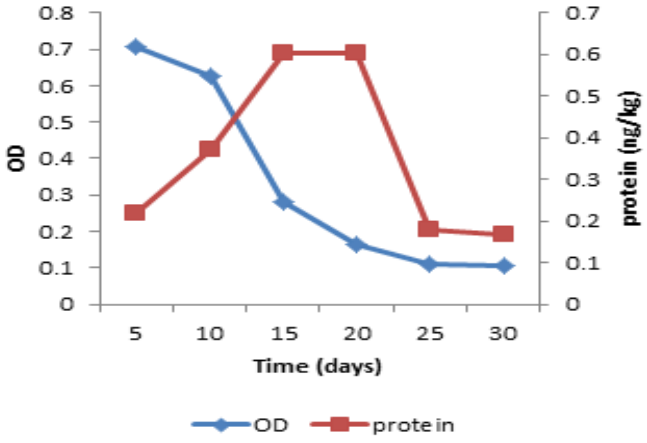

Figure 2: Growth and degradation of heptachlor by bacteria isolates grown in MM after 30 days of cultivation

Metabolite identification

Figure 3 shows retention time the different component of Citrobacter sp extract cultured for 30 days in MM. Gas chromatography revealed three peaks (retention times 8.74; 9.28 and 10.78 minutes).

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Figure 4 shows mass spectra and the scheme of a metabolite that occurs at minute 10.78, identified as 1-hydroxichloredene, which belongs to a metabolite resulting from heptachlor hydrolysis. And one peak unknown at retention times 9.28 minute was consistent from all MM.

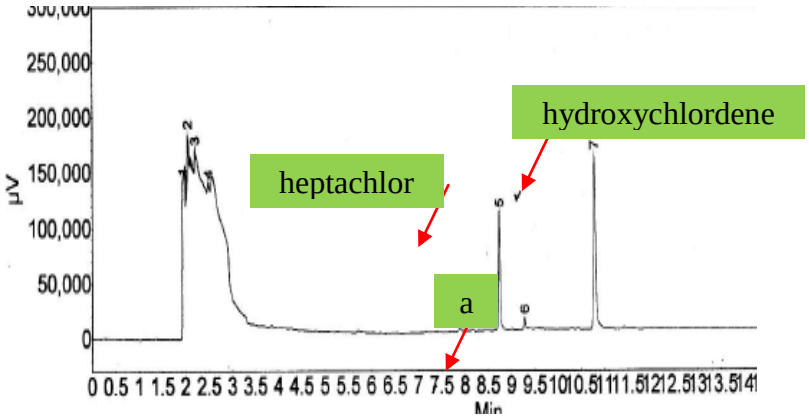

Figure 3: Histogram heptachlor metabolite 1-hydroxychlordene and unknown.

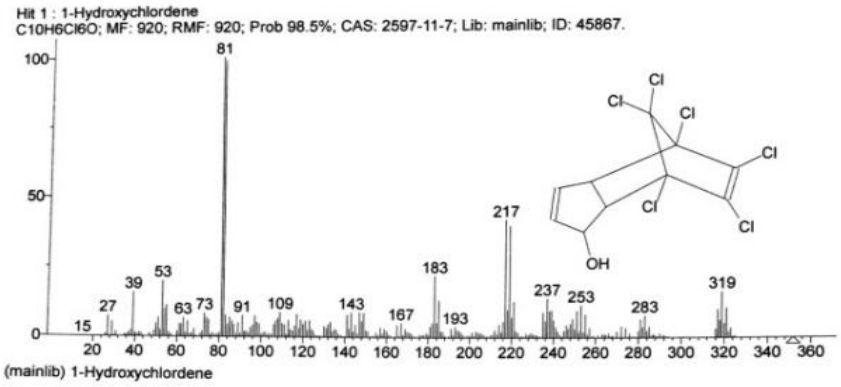

Figure 4: Chemical structure and mass spectra of metabolite 1-hydroxychlordene

Morphological characteristics of *Citrobacter* heptachlor degrading bacteria were determined by observation of colony morphology, Gram's straining and sell morphology under a microscope at 1000x magnification. *Citrobacter* were non motile aerobic facultative bacteria, Gram-negative, and bacillus. Since isolate Citrobacter appeared to have the higest degradation ability at 50% and sufficient growth/or degradation rates within 30 days, it was selected for further optimazion studies indicated that this isolate has 99% identity with *Raoultella ornithinolytica* B4**.**

Raoultella ornithinolytica B4 is pathogenic bacterium, which *Raoultella* isolated from clinical specimens has many virulence factors such as capsule, colonization factors antigens (CFA/I and CFA/III), production of siderophore, histamine and bacteriocin.

CONCLUSION

Biotranformation of heptachlor become metabolite was 1-hydroxychlordene by R. ornitolytica B4. The bacteia whose patogentic on human skin.

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