Makara Journal of Science

Volume 23
Issue 4 *December*

Article 6

12-20-2019

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Recommended Citation

Indratin, Indratin; Kurnia, Asep; and Wahyuni, Sri (2019) "Degradation of Cypermethrin by Indigenous Bacteria from Contaminated Soil," *Makara Journal of Science*: Vol. 23 : Iss. 4 , Article 6.
DOI: 10.7454/mss.v23i4.7998
Available at: https://scholarhub.ui.ac.id/science/vol23/iss4/6

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Cover Page Footnote

We gratefully acknowledge the Indonesian Agency for Agricultural Research and Development for providing funding for this work (Penelitian APBN 2017). We also thank all of the laboratory staffs who contributed their time and knowledge.

Makara Journal of Science, 23/4 (2019), 210-216
doi: 10.7454/mss.v23i4.7998

Degradation of Cypermethrin by Indigenous Bacteria from Contaminated Soil

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Received June 20, 2019 | Accepted August 13, 2019

Abstract

Soil contamination by pesticides should be controlled by using soil microbes with the ability to degrade pesticide residues. Microorganisms that have adapted to a particular pesticide could accelerate the degradation process. The present study aimed to select bacteria in soil that could potentially degrade cypermethrin residues. Experiments were carried out in a laboratory and employed soil samples collected from the districts of Demak, Magelang, and Brebes. Each soil sample was initially tested for the presence of bacteria and pesticide residues. Indigenous microbes capable of living in contaminated environments were adapted to cypermethryn and cultured in the laboratory. Experiment was carried out in several stages, namely, (1) isolation and identification of microbes that could degrade cypermethrin, (2) testing of the growth characteristics of isolates in cypermethrin, and (3) determination of cypermethrin residues in cultures. Three isolates, namely *Pseudomonas alcaligenes*, *Bacillus amyloliquenfaciens*, and *Pseudomonas aeruginosa*, were found to decrease cypermethrin residues by up to 95% with fast half-lives and good growth capability. The results demonstrate the potential applications of the isolates in biodegradation processes and remediation of cultivation fields.

Keywords: degradation, cypermethrin, contaminated soil

Introduction

Cypermethrin (IUPAC name: cyano-(3-phenoxyphenyl) methyl]3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate) is a synthetic pyrethroid insecticide widely used in agriculture and the environment. It is used in agriculture to control insects in vegetable crops, fruit, paddy, cereal, cotton, ornamental plants, and treatment of cattle diseases. In the environment, including households, it is used to control ants and cockroaches [1-3].

Cypermethrin is a neurotoxic insecticide that acts on the central nervous system to produce a hyperexcitable state by interacting with sodium channels. Besides it affects voltage-dependent sodium channels and ATPase system in neuronal membranes that binds to nuclear DNA, it also leads to destabilization and unwinding of DNA [4,5]. Cypermethrin is a broad-spectrum insecticide that kills both beneficial and target insects. Intensive use of cypermethrin could lead to insect resistance and causes environmental damage and adverse effects on human health [6,7]. It is highly toxic to fish and aquatic invertebrates and causes low levels of red blood cells and blood proteins [3,8-13]. Moreover, it has been classified as a possible human carcinogen. Cypermethrin is known to cause allergic skin reactions, eye irritation in humans [9,14,15], neurotoxicity [16], immunotoxicity [17], genotoxicity [18], reproductive toxicity [19], and endocrine disruption effects [20].

Cypermethrin has been used in agriculture for over 40 years [21,22]. Indeed, cypermethrin application has increased annually due to as substitution of some organophosphate pesticides that have been restricted [23]. When applied to crops, some of the pesticide drops into the soil, where it is infiltrated and leached by water and carried to rivers and lakes [24,25]. Previous research detected cypermethrine in vegetables and fruits [26,27], as well as in soil, downstream watersheds, and sediments due to run off and erosion from agriculture and nonagricultural application [28-32]. Thus, developing a method to decrease cypermethrin residues in the environment is necessary.

Cypermethrin could be degraded by using microbes such as bacteria and fungi. Microbial degradation is the most efficient way to decrease of numerous xenobiotic/ recalcitrant compounds, including cypermethrin [34], in a contaminated environment [33]. Several bacteria, such as *Pseudomonas* sp. [35,36], *Micrococcus* sp. [9], *Serratia* sp. [15,35], *Streptomyces* sp. [1], and *Ochrobactrum* sp. [37], have been reported to degrade cypermethrin. The most effective application of microbial degradation involves the use of microbial

consortia rather than a single microbial species [36,38,41,42]. Microbial consortia could exert synergistic effects on pesticide degradation in which one to another species will utilize carbon and another element of pesticides more plentyfull than single microbe [1].

Many indigenous bacteria in soil may be capable of pesticide biodegradation. Thus, the present study aimed to determine bacteria that could degrade cypermethrin residues in soil.

Materials and Methods

Soil. The soil used in this experiment was collected from three different agricultural fields in the districts of Demak, Magelang, and Brebes, Central Java Province, Indonesia all of which have a history of cypermethrin and other pesticide application spanning over 10 years. Demak is one of the largest paddy fields in this province, while Magelang and Brebes are the largest areas for cultivating red chili and onion, respectively. The soil type in Demak and Brebes is inceptisol, while that in Magelang is andosol. One-kilogram soil samples were obtained from each location by compositing from five collection points at a depth of 10 − 20 cm, transferred to sterile plastic bags, and stored at 4 °C. The soil was dried and sieved (2 mm mesh) prior to use [34].

Chemicals and Media. Cypermethrin standard with purity 99.4% was obtained from Chemservice (USA). Analytical-grade acetone, *n*-hexane, alcohol, dichloromethane, anhydrous sodium sulfate, and potassium hydroxide were obtained from Merck (Germany). Cellite 545, nutrient agar (NA), NB medium, nitrate mineral salts (NMS) medium, and filter paper were obtained from Sigma Aldrich (Japan). A 1000 ppm of cypermethrine standard stock solution was diluted used acetone.

Microbial isolations were carried out in NMS medium with the following composition: $MgSO_4 \cdot 7H_2O$ 1.0 g/L; $CaCl_2 \cdot 6H_2O$ 0.2 g/L; KNO_3 1.0 g/L; KH_2PO_4 0.272 g/L; Na_2HPO_4 4.0 g/L; NH_4Cl 4.0 mg/L; Na_2EDTA 0.5 g/L; $FeSO_4 \cdot 7H_2O$ 0.2 g/L; H_3BO_4 0.03 g/L; $CoCl_2 \cdot 6H_2O$ 0.02 g/L; $ZnSO_4 \cdot 7H_2O$ 0.01 g/L; $MnCl_2 \cdot 4H_2O$ 3.0 mg/L; $Na_2MoO_4 \cdot 2H_2O$ 3.0 mg/L; $NiCl_2 \cdot 6H_2O$ 2.0 mg/L; $CaCl_2 \cdot 2H_2O$ 1.0 mg/L) [43].

Initial Soil Bacterial Population. Initial soil bacterial populations were tested by using a bacterial SANI-Check kit [39] according to the manufacturer's instructions. Briefly, the vial cap was unscrewed, and the paddle was carefully removed from the vial to avoid contamination. The paddle was immersed into the sample for 2−3 s so that both surfaces were covered with the liquid. The excess fluid was allowed to drain from the paddle by touching the tip of the latter to an absorbent piece of paper, such as a paper towel. The paddle was returned to the vial, and the cap was screwed back on. The vial was incubated in an upright position at 25−30 °C for 24−48 h for bacterial growth. Finally, the growth on the paddle was compared with that on a conversion chart (Figure 1) to quantify the results.

Enrichment, Isolation, and Screening of CP-degrading Strains. For strain enrichment, soil samples (5 g) were added with 100 mL of NMS supplemented with 10 mg L^{-1} cypermethrin in 250 mL Erlenmeyer flasks and then incubated at 150 rpm and 30 °C [34]. The rest of the procedure was carried out as described by Chen et al. [40]. Ten-fold-diluted cultures were prepared from the last enrichment culture, and 100 µL of each dilution was spread onto minimal agar plates containing cypermethrin (10 mg L^{-1}). Isolated colonies were subsequently purified. Screening for cypermethrin degradation capacity was carried out by streaking NMS gradient plates, and isolates showing heavy growth at higher concentrations were selected. The selected isolates were then cultured in NMS containing 10 mg L^{-1} cypermethrin for 1 week, and residual cypermethrin concentrations were determined by GC. Seven isolates with the highest cypermethrin degradation potential, designated as S-50, S-56, S-58, S-97, S-169, S-102, and S-167, were selected for further studies.

Biodegradation of Cypermethrin in Liquid Medium. Seed cultures of each isolate were grown in nutrient broth containing 10 mg L^{-1} cypermethrin, harvested by centrifugation at 4,600 rpm, washed, and diluted with Milli-Q H_2O to achieve an optical density at 600 nm (OD_{600}) of 1.0. Exactly 2% of this suspension was used as the inoculum. Erlenmeyer flasks (250 mL) containing NMS (100 mL) amended with 10 mg L^{-1} cypermethrin were inoculated with the bacterial cell suspensions in triplicate. The flasks were incubated at 30 °C with shaking at 150 rpm, and an uninoculated flask was used as the control. Every 6 h until 96 h, 10 mL of cultures from the control and treated flasks was removed and centrifuged. The supernatant was extracted with ethyl acetate (20 mL × 3), and the pooled organic phase was dried over anhydrous sodium sulfate and evaporated at 40 °C under vacuum. The residue was dissolved in

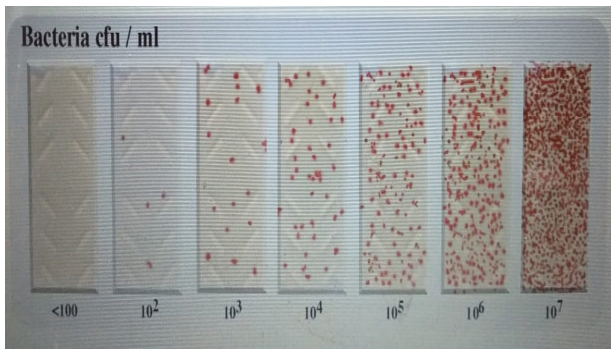

Figure 1. Bacteria Population Conversion Chart

acetone, and the final volume was made to 10 mL for GC analysis. For growth studies, 1 mL of culture was withdrawn every 6 h until 96 h, and growth was evaluated as OD_{600} [34].

Data Analysis. The cypermethrin degradation rate constant (k) was determined using the kinetic model $C_t = C_0 \times e^{-kt}$, where C_0 is the initial concentration at time zero, C_t is the concentration at time t, t is the degradation period in days, and k is the rate constant (d^{-1}). The half-life ($T_{1/2}$) of cypermethrin was determined using the equation $T_{1/2} = \ln 2/k$. Correlation coefficients (R^2) and regression equations were calculated from the linear relation between $\ln(C_t/C_0)$ of chemical data with observation time [39].

Chemical Analysis. Analysis of cypermethrin residues in the collected soil samples was performed according to Anastassiades et al. [44]. Residual pesticide was extracted by adding 5 mL of culture broth to 20 mL of acetone in a flask. The mixture was shaken for 1 h and filtered using a Buchner funnel The obtained residue was thoroughly washed with 10 mL of acetone and filtered once more. The filtrate was collected in a round-bottom flask [33].

Gas Chromatography Analysis. Cypermethrin was analyzed on a Varian 450 GC equipped with a thermionic specific detector. Nitrogen was used as the mobile phase, and C18 was used as the stationary phase. The injector, column, and detector temperatures were 250, 230, and 250 °C, respectively. Data processing was using Galaxy Software System. The injection volume was 10 µL, the analysis time was 20 min, and the flow rate was 1 mL min^{-1}. The retention time of cypermethrin was 3.94 min. The calibration curves of cypermethrin constructed from serial dilutions of the samples dissolved in 100% acetone. The linear regression equation were obtained sequentially at 0–1 ppm at intervals of 0.2 and peak areas.. Mean areas generated from the standard solution were plotted against concentration to establish a calibration equation, and the concentration of cypermethrin was determined on the basis of the peak areas in the chromatograms.

DNA Sequence and Phylogenetic Analysis. Each 50 µL PCR micro-tube contained 2 µL of purified extracted DNA, 1 µL of the upstream primer (10 pmol), 1 µL of the downstream primer (10 pmol), 5 µL of 10×Ex Taq buffer, 0.5 µL of Ex Taq DNA polymerase , 4 µL of 2.5 mM dNTP and 36.5 µL of sterile MQ water. DNA amplification was performed in a thermal cycler with the following conditions: denaturation for 3 min at 94 °C; 35 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1.5 min; and extension of 72 °C for 5 min [1]. BLAST search was carried out at the National Center for Biotechnology Information.

Results and Discussion

Initial Soil Bacterial Population. The SANI-Check kit results showed that the initial soil bacterial population was 10^2−10^7 cfu/ml. The lowest bacterial population density was observed in soil from Magelang, which could be explained by soil sampling within a short time after pesticide spraying (within 2 h), which kills bacteria. The results of SANI-Check are presented in Table 1.

Isolation and Screening of Cypermethrin-degrading Strains. Isolation is the first step of bacterial exploration. Bacterial isolation phase 1 involved cultivation of soil bacteria taken from each location via the scratch cup and saucer method, which yielded 196 isolates. Phase 2 involved selection of the best-growing isolates, which yielded seven isolates. The selected isolates were then purified 1−3 times to obtain pure isolates.

A range of morphologically different bacterial strains were isolated from the enrichment culture, and these strains were grown on NMS medium containing up to 100 mg L^{-1} cypermethrin. Isolates showing effective growth on the gradient plates were selected. Screening tests based on cypermethrin degradation capacity showed that the isolates were able to degrade 46%–75% of the applied cypermethrin (100 mg L^{-1}). Maximum potential was shown by S-50, which degraded 75% of the available cypermethrin. The relevant results are presented in Table 2.

Table 1. Initial Soil Bacteria Population

No.	Location	Vegetation	Bacteria Population (cfu/mL)
1	Demak	X	10^6
2	Magelang 1	Y	10^2
3	Magelang 2	Z	10^7
4	Brebes	R	10^7

Table 2. *In vitro* Degradation of Cypermethrin by the Selected Bacterial Isolates After 12 h

No.	Pesticide Code	Decrease of Cypermethrin (%) *
1	S-50	75
2	S-56	73
3	S-58	73
4	S-97	72
5	S-169	70
6	S-102	57
7	S-167	46

*Values are averaged from three replicates

Bacterial Growth and Biodegradation of Cypermethrin in Nitrate Mineral Salts Medium. Three isolates with good growth capability, namely, S-50, S-58, and S-97, were chosen, and degradation was carried out in liquid culture containing cypermethrin for 0, 24, 48, 72, and 96 h. Isolate S-58 was able to decrease the concentration of cypermethrin from 10.2 ppm to 2.2 ppm (78%) within 24 h. Within 96 h, isolate S-58 was able to decrease the cypermethrin concentration from 10.2 ppm to 0.5 ppm (95%). Such degradation ability was followed by that of isolate S-97, which could decrease cypermethrin concentration by 78% within 24 h and 94% within 96 h. The relevant results are presented in Table 3.

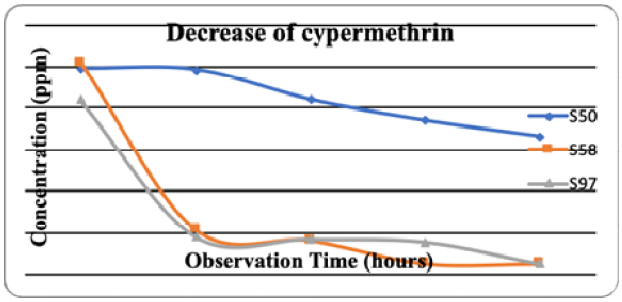

Figure 3. Decrease in Cypermethrin Concentration Under the Action of Three Selected Bacterial Strains

Table 3. First-order Kinetic Parameters of the Degradation of Cypermethrin

Treatments	Regression Equation	k (h^{-1})	$t_{1/2}$ (h)	R^2
S-50	$C_t=9.899e^{-0.000236t}$	0.00024	2931.77	0.9930
S-58	$C_t=10.198e^{-0.063898t}$	0.06389	10.85	0.8789
S-97	$C_t=8.389e^{-0.063181t}$	0.06318	10.97	0.7043

Note: C_t = cypermethrin concentration (mg L^{-1}); t = degradation period (h); R^2= correlation coefficient

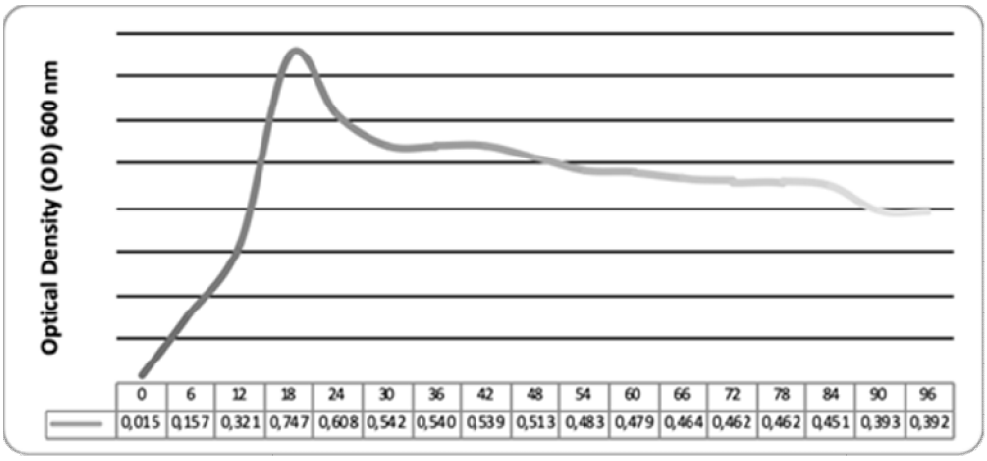

Figure 4. Growth Curve of Bacterial Isolate S-97

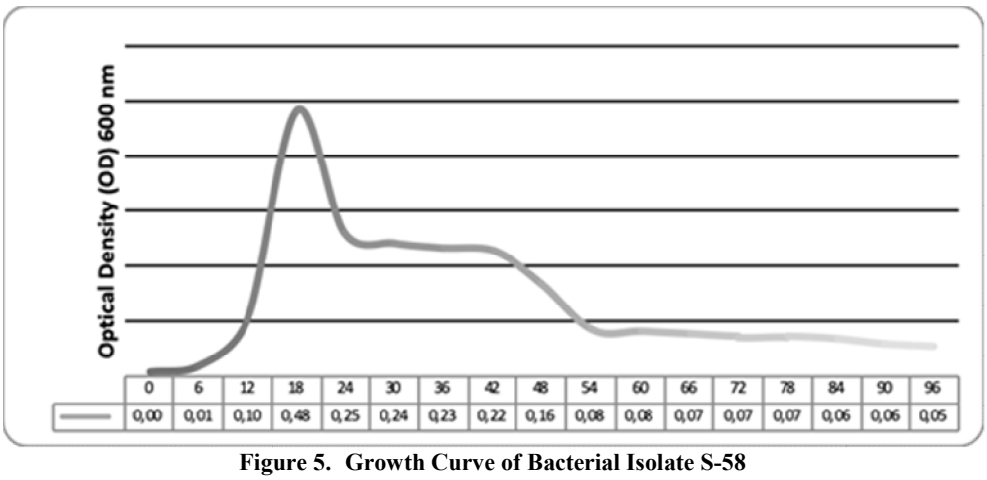

Figure 5. Growth Curve of Bacterial Isolate S-58

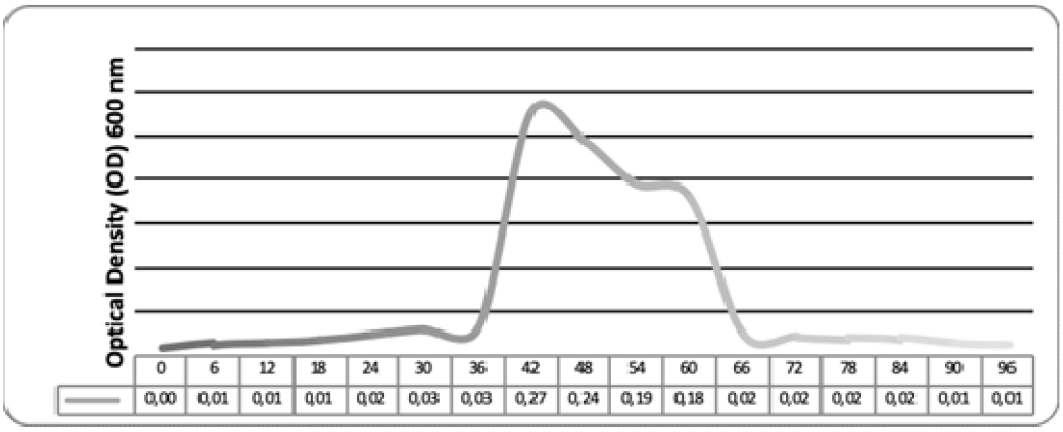

Figure 6. Growth Curve of Bacterial Isolate S-50

Table 4. Gram (+) and (−) Results of the Three Isolates

Isolate Number	Isolate Code	Gram Test Result
1	S- 50	Gram (−)
2	S- 58	Gram (+)
3	S- 97	Gram (−)

Biodegradation kinetics showed that the degradation process corresponds to first-order kinetics. R^2 values ranged from 0.7043 to 0.9930, which indicates the good fit of the data to the model. The k values of S-50, S-58, and S-97 were 0.00024, 0.06389, and 0.06318 h^{-1}, respectively. The half-lives of cypermethrin under the action of S-50, S-58, and S-97 were 2931.77, 10.85, and 10.97 h, respectively. These results reveal the high cypermethrin degradation efficiency of S-58 and S-97. S. Akbar [34] reported that the half-lives of cypermethrin under bacterial degradation range from 9.6 days to 101 days. The first-order kinetic parameters for all runs are summarized in Table 3.

Growth is a process in which the cells of an organism increase their mass. Microorganisms such as bacteria have several growth phases, including the lag phase, log phase, stationary phase, exponential phase, and death phase. In this study, the growth rate of the selected isolates was tested. Growth observations were performed every 6 h by measuring OD_{600}. The results showed at figure 4, 5 and 6.

Molecular Identification of Bacteria. Prior to bacterial DNA extraction, the isolates were rejuvenated in media NB100%. The Gram (−)/(+) results of the three isolates are shown in Table 4.

Isolate S-50 was identified as *Pseudomonas alcaligenes* with 99% homology, isolate S-58 was identified as *Bacillus amyloliquenfaciens* with 99% homology, and isolate S-97 was identified as *Pseudomonas aeruginosa* with 100% homology. The high homology values showed that the isolates are truly similar to the comparing isolates.

Conclusion

Three isolates, namely *P. alcaligenes*, *B. amyloliquenfaciens*, and *P. aeruginosa*, could decrease cypermethrin residues by up to 95% with good growth capability. The findings demonstrate the potential applications of these species in biodegradation processes and remediation of cultivation fields.

Acknowledgments

We gratefully acknowledge the Indonesian Agency for Agricultural Research and Development for providing funding for this work (Penelitian APBN 2017). We also thank all of the laboratory staffs who contributed their time and knowledge.

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