Isolation and identification of profenofos pesticide degrading bacterium from soil sample of Bedugul, Indonesia

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Abstract. Climate change might change pest populations and in certain circumstances can increase the use of pesticides and the residue on the soil also increases. To reduce the negative impact of the residue, pesticide degrading bacteria are needed. This study aimed to isolate and identify profenofos-degrading bacterial strains from the soil that has been contaminated by profenofos pesticides for many years. The bacterial strain was isolated from a long term profenofos exposed soil by an enrichment technique, and its ability to degrade profenofos was determined using gas chromatography. The results were obtained 8 isolates from 9 sampling points. Isolates have different abilities to degrade profenofos especially isolated BS-06 had the highest degradation rate of profenofos was identified as Pseudomonas luteola according to its physiological and biochemical properties (API 20E system). The isolate has a rod shape, motile, Gram-negative, and has an optimum pH in degrading profenofos at 7. The degradation of profenofos was examined using a medium treated with 100 mg/L profenofos, which resulted in a higher degradation rate than the control without inoculation. In a mineral salt medium (MSPY), removal of the level of profenofos of 92.72% was obtained within 54 h of incubation.

1. Introduction

Millions of tons of pesticides were applied manually, but it was believed that only a small fraction of these products effectively reaches the target organisms, and the remainder are deposited on the soil. The persistence of pesticides in soil affects significant climate factors that as intensity and timing of rainfall and temperature. Currently, there has been climate change can affect the persistence of pesticides in soil and microbes. However, while pesticides may have a beneficial effect on agricultural productivity, their indiscriminate use causes many serious problems to the environment and human health, since these compounds are toxic to non-target species [1].

Bedugul is commonly known as the cultivation area and the producing of high vegetables and fruits in Bali - Indonesia. The development of horticultural cultivation has been socialized by governments and colleges directly through the field plot area that can be seen directly by farmers. One of the rice fields has been used to know the process of bioremediation in situ with a variety of doses spraying pesticides [2].

Horticultural cultivation cannot separate from supporting tools, fertilizer, and pesticides to maximize the cultivation result of agriculture. One kind of pesticide used in the field of agriculture is profenofos insecticide organophosphate (OPs) [3]. Profenofos used to control P. operculella, Thrips sp. on potatoes; C. pavonana, P. xylostella on cabbage; and H. armigera in tomatoes [4]. Profenofos has an effective
and rapid nature of killing resistant insects, but the profenofos are highly toxic and can kill non-target organisms [5][6].

One of the most widely accepted impacts of pesticide residue is soil. Microscopic and macroscopic organisms will have a deterioration of adapting to pesticide residues in soil [7]. The main group to utilize pesticide residue as a carbon source on the soil is bacteria. The ability of bacteria to utilize pesticide residue on soils is very significant. Some bacteria capable to utilize residual pesticides in the soil are Lysinibacillus fusiformis ADI-01, Pseudomonas pseudoalcaligenes ADI-03, Pseudomonas pseudoalcaligenes ADI-06, Bacillus cereus ADI-10 [8], Vibrio metschinkouii, Serratia ficaria, Serratia spp., Yersinia enterocolitica in utilizing tetrachlorvinphos [9].

The degradation ability of bacteria to degrade organophosphates is well established and researchers have even proposed possible degradation mechanisms for the OPs [10]. Several results that bacterial were degraded profenofos 97% within 96 h [3] and up to 500 mg/L without the addition of any extra carbon source [8].

In this study, the research aims to isolate the bacteria which the highest ability to degrade the profenofos by taking soil samples that have been contaminated with pesticides from a long time ago. Furthermore, one selected strain was identified (selected which highest ability degrade profenofos), determination growth pattern, and optimum pH.

2. Materials and methods

2.1. Medium for isolation
Selective medium mineral salt peptone yeast (MSPY) agar was made by dissolved in 1 L: 0.2 g KH₂PO₄; 0.5 g K₂HPO₄; 0.2 g MgSO₄·7H₂O; 0.2 g NaCl; 0.05 g CaCl₂·2H₂O; 0.025 g FeSO₄·7H₂O; 0.0005 g MnSO₄; 1.0 g peptone; 2.0 g yeast extract; 1.5 g bacto agar and adjusted to pH 7.0 (neutral). Then sterilized at 121°C for 15 min [11][12]. The medium was cooled down around ±40°C, and then supplemented with 100 mg/L profenofos (Curacron 500 EC) as the sole carbon source. The MSPY broth was made without agar.

Table 1. Properties of the soil samples for chemical analysis.

| Locations soil sample                  | Sample No. | pH  | Organic matter (g/kg) | Sand (%) | Silt (%) | Clay (%) |
|---------------------------------------|------------|-----|-----------------------|----------|----------|----------|
| Br. Mayungan, Antapan Village         | 1          | 6.6 | 52                    | 35       | 38       | 27       |
|                                       | 2          | 6.7 | 51                    | 34       | 37       | 29       |
|                                       | 3          | 6.5 | 53                    | 38       | 42       | 20       |
|                                       | 4          | 6.4 | 54                    | 34       | 40       | 26       |
|                                       | 5          | 6.6 | 52                    | 37       | 37       | 26       |
| Br. Pemuteran, Candikuning Village    | 1          | 6.6 | 58                    | 42       | 33       | 25       |
|                                       | 2          | 6.7 | 57                    | 43       | 37       | 20       |
|                                       | 3          | 6.7 | 59                    | 44       | 32       | 24       |
|                                       | 4          | 6.6 | 60                    | 40       | 36       | 24       |
|                                       | 5          | 6.6 | 55                    | 43       | 37       | 20       |
| Br. Bukit Catu, Candikuning Village   | 1          | 6.7 | 56                    | 44       | 35       | 21       |
|                                       | 2          | 6.7 | 57                    | 43       | 37       | 20       |
|                                       | 3          | 6.7 | 54                    | 44       | 34       | 22       |
|                                       | 4          | 6.6 | 58                    | 43       | 36       | 21       |
|                                       | 5          | 6.8 | 56                    | 43       | 37       | 20       |

2.2. Sample collection
The soil sampling location at one of the field plot area in Candikuning Bedugul. Based on the results of interviews with the owner, there are several types of pesticides used that are a fungicide (Daconil), a herbicide (Sidastar), and an insecticide (Curacron). The most dominant type of pesticides used is an
insecticide (Curacron 500 EC, containing profenofos as an active compound). Profenofos as an organophosphorus pesticide has been extensively used to control insect populations. Soil samples were collected with simple random sampling at 9 points with ± 5-10 cm depth [13] and with a history of 7-9 years of profenofos applications. The collected samples were stored at 4°C. Soil sample properties are shown in Table 1.

2.3. Isolation, screening, and identification
The samples were made diluted until 10^-5. First, a 25 g sample was placed in 225 mL sterile saline solution (NaCl 0.85%) and then 1 mL of suspension was transferred into a fresh 9 ml saline solution until dilution 10^-5. The suspension in dilution 10^-3 to 10^-5 was inoculated in MSPY agar with the spread plate technique. Inoculated plates were incubated at 37°C for 48 h [12][14]. The colonies that grew with different characteristics were streaked 2 times with the quadrant streak technique into fresh MSPY agar to obtain a pure strain.

All strains were transferred into MSPY broth to produced widely cells by multilevel sections at 30°C for 48 h with shaking at 100 rpm (first enrichment). Then, all first enrichments were inoculated into fresh MSPY broth with wide volume (second enrichment) and incubated with the same method. Cells were harvested by centrifugation with the speed of 5,000 rpm for 15 min in 4°C and washed until 2 times by adding saline solution. Adjust cells as the optical density by measuring in spectrophotometer UV-Vis at wavelengths 660 nm (OD_{660}) to absorbance 5 [15]. Cells were inoculated in MSPY broth and incubated at 30°C for 48 h with shaking at 100 rpm. After incubation, growth was measured at OD_{660}, pH and profenofos residues were analyzed. Profenofos residues were analyzed before and after incubation as a comparator to determine 1 strain which the highest degradation capability to utilize profenofos as a carbon source, was selected for further studies. Profenofos residues were analyzed with Gas Chromatography (Agilent Technologies 6890N) with Mass Selective (MS) detector [12].

The selected strain was identified as morphological (cell forms, Gram staining, and motility) [14] and biochemical tests according to Keletso and Olubukola [16] that use the analytical profile index test (API 20E). The results were incorporated into the Web API program/software and bacteria species can be known at the output of the program.

2.4. Determination growth pattern of a selected strain
This step was the same as a screening method. After adjusting the cells, the cells were inoculated in MSPY broth and then incubated at 30°C with 100 rpm shaking. Growth (OD_{660}) and pH during incubation were monitored every 2 h. Profenofos residues were analyzed shortly after the lag phase, the log phase and the stationary phase. The phase which the highest degradation capability as the optimal time for incubation was used for further incubation time.

2.5. Effect of pH on degradation activity
Cells were inoculated in MSPY with pH variations 6, 6.5, 7, 7.5, 8, and pH 7 used as a control without cell [12] and incubated with the time was determined of growth conditions. After incubation, OD_{660} was measured as a growth, pH and profenofos residues were analyzed. The pH which the highest degradation capability as optimal pH was used for further studies.

2.6. Gas chromatography analysis
The highest concentration of the profenofos and metabolite (4-bromo-2-chlorophenol) was in the liquid medium [17]. Profenofos and metabolite were extracted with hexane solvent and analyzed using GC-MS with Nitrogen as a carrier gas, and HP-5MS column (length 30 m x diameter 0.32 mm). The temperature of the injector was set at 250°C, the initial temperature of the oven column was set at 160°C, with the rate of temperature rise 10°C/min and an oven's final temperature at 270°C.
3. Result and discussion

3.1. Isolation, screening, and identification

After 48 h completion, differentiated colonies were noted, colored, 1 mm to 3 mm in diameter. A total of 8 bacterial isolates were obtained from soil samples and the degradation ability of profenophos was studied and compared. Characteristics of bacterial isolates are shown in Table 2.

| Isolates | Characteristics of bacterial isolates |
|----------|--------------------------------------|
|          | Form | Diameter (mm) | Color | Margin |
| BS-01    | Circular | 1.2 | Yellow | Entire |
| BS-02    | Circular | 1.5 | White  | Entire |
| BS-03    | Circular | 2.2 | Green  | Entire |
| BS-04    | Circular (clear zone) | 2.1 | Invisible white | Undulate |
| BS-05    | Circular | 2.6 | Pale yellow | Lobate |
| BS-06    | Irregular | 3.1 | White  | Entire |
| BS-07    | Circular | 2.4 | White  | Undulate |
| BS-08    | Circular | 2.7 | Cream  | Entire |

*MSPY agar medium supplemented with 100 mg/L profenofos and incubated at 30°C for 48 h with shaking at 100 rpm.

In this screening experiment, all isolates showed that they had degradation ability. These indicated strains were successfully utilized the profenofos as a carbon source, even though were not until 100% degradation. Carbon is one of the most important elements for microbial growth, as carbon compounds provide energy for cell growth and serve as the basic units to build cell materials. Nitrogen is also essential to the organisms, as well as other elements (hydrogen, oxygen, and phosphorus). Thus, organism growth in the presence of pesticides may indicate tolerance to the pesticide toxicity; pesticide metabolism as a mechanism of defense of the microorganism to eliminate the xenobiotic compound; or even pesticide use as a source of nutrient for growth, since the organophosphate pesticide profenofos has carbon, oxygen, sulfur, and phosphorus in its structure [17][18]. The data screening strain is shown in Table 3.

| Isolate codes | Analyzed before incubation | Analyzed after incubation |
|---------------|----------------------------|---------------------------|
|               | OD_{660} | pH | Profenofos residues (mg/L) | OD_{660} | pH | Profenofos residues (mg/L) | Profenofos degradation rate (%) |
| Control b     | 0.02     | 6.97 | 97.73 | 0.02 | 6.93 | 97.67 | 0.06 |
| BS-01         | 0.27     | 6.99 | 97.73 | 1.98 | 7.95 | 10.97 | 88.77 |
| BS-02         | 0.27     | 7.00 | 97.73 | 1.71 | 7.67 | 16.66 | 82.95 |
| BS-03         | 0.28     | 7.00 | 97.73 | 2.02 | 7.93 | 45.69 | 53.23 |
| BS-04         | 0.27     | 6.99 | 97.73 | 2.05 | 7.94 | 81.88 | 16.19 |
| BS-05         | 0.27     | 7.00 | 97.73 | 1.63 | 7.67 | 33.59 | 65.62 |
| BS-06         | 0.27     | 6.99 | 97.73 | 1.92 | 7.94 | 7.11 | 92.72 |
| BS-07         | 0.27     | 6.99 | 97.73 | 1.75 | 7.97 | 41.65 | 57.36 |
| BS-08         | 0.27     | 7.00 | 97.73 | 2.03 | 8.00 | 8.63 | 91.16 |

※MSPY broth medium supplemented with 100 mg/L profenofos and incubated at 30°C for 48 h with shaking at 100 rpm.

bControl was incubated without culture bacterium.
The degradation ability showed that the BS-06 isolate had the highest degradation rate of profenofos until 92.72% but was not accompanied by the highest growth rate. Strain BS-06 was selected based on the highest degradation rate for further study. It was possible to suggest that these strains showed good potential for biocatalytic degradation of profenofos. Strain BS-06 morphological and biochemical characteristics are presented in Table 4.

Table 4. Morphological and biochemical characteristics of strain BS-06*

| Characteristics                        | Strain BS-06   |
|----------------------------------------|----------------|
| Morphological characteristics          |                |
| Cell type (shape)                      | Rods           |
| Gram staining                          | Negative       |
| Motility                               | Motile         |
| Biochemical characteristics            |                |
| 2-Nitrophenyl-βD-galactopyranoside     | -              |
| L-Arginine                             | +              |
| L-Lysine                               | -              |
| L-Ornithine                           | -              |
| Trisodium citrate                     | +              |
| Sodium thiosulfate                     | -              |
| Urea                                   | -              |
| L-Tryptophan                           | +              |
| L-Tryptophan                           | -              |
| Sodium pyruvate                        | +              |
| Gelatin                                | -              |
| D-Glucose                              | +              |
| D-Mannitol                             | -              |
| Inositol                               | -              |
| D-Sorbitol                             | -              |
| L-Rhamnose                             | -              |
| D-Sucrose                              | -              |
| D-Melibiose                            | -              |
| D-Amygdalin                            | -              |
| L-Arabinose                            | -              |
| Name of strain BS-06                   | Pseudomonas luteola |

*Biochemical test was used kit API-20E, (+): positive, (-): negative.

*Pseudomonas* sp. has been widely discovered by researchers in capability degraded pesticides and heavy metals. Chaudhry *et al.* [19] used *Pseudomonas* sp. as a mixed culture to degraded parathion and metal parathion which indicated that mixed cultures are more stable in maintained their ability to decreased methyl parathion by utilizing it as a carbon source. *Pseudomonas* was capable of degrading metal parathion by making it a source of carbon and phosphorus sources [5][9][20]. In addition, *Pseudomonas* genus has been found in the ability to degrades heavy metals such as chromium, lead, cadmium, zinc, and nickel [21].

3.2. Growth pattern of the selected isolate
The incubation time was found 54 h with a degradation rate of profenofos 76.82%. This time was used for further study. Growth pattern, pH change, and degradation rate of profenofos by isolate BS-06 during incubation are shown in Figure 1.
Figure 1. Growth pattern of *Pseudomonas luteola* strain BS-06 in MSPY broth medium supplemented with 100 mg/L profenofos and incubated at 30°C for 48 h with shaking at 100 rpm.

3.3. Effect of pH on degradation activity

The pH was found at 7 (neutral) with a degradation rate of profenofos 88.96%. This result was similar to Chaudhry et al. [19] which obtained microbial degradation organophosphate pesticides had pH range in 7 to 9.5 as the optimum pH. pH plays an important role in membrane-bound proton pumps and protein stability, and thus directly imposes a physiological constraint on microorganisms. Table 5 showed the effect of pH on the degradation activity of *P. luteola* strain BS-06.

Table 5. Effect of different pH towards growth and degradation profenofos by *P. luteola* strain BS-06.

| pH  | Analyzed before incubation | Analyzed after incubation |
|-----|----------------------------|---------------------------|
|     | OD_{660} | pH | Profenofos residues (mg/L) | OD_{660} | pH | Profenofos residues (mg/L) | Profenofos degradation rate (%) |
| Control⁵ | 0.02  | 6.98  | 98.78 | 0.03  | 6.99  | 98.46 | 0.32 |
| 6    | 0.27  | 6.14  | 98.78 | 1.18  | 8.07  | 70.08 | 28.94 |
| 6.5  | 0.27  | 6.59  | 98.78 | 1.43  | 8.07  | 72.48 | 26.50 |
| 7    | 0.27  | 7.03  | 98.78 | 1.74  | 8.02  | 10.89 | 88.96 |
| 7.5  | 0.28  | 7.47  | 98.78 | 1.42  | 8.23  | 23.23 | 76.44 |
| 8    | 0.27  | 7.96  | 98.78 | 1.13  | 8.37  | 19.41 | 80.31 |

⁵MSPY broth medium supplemented with 100 mg/L profenofos and incubated at 30°C for 54 h with shaking at 100 rpm.

Conclusion

The results of this study showed that there were microbes that can survive climate changes and could degrade the toxic insecticide organophosphate profenofos compounds. Isolates had different abilities to
degrade these compounds, BS-06 isolate which had the highest ability to degrade profenophos was identified as *Pseudomonas luteola*.

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