Isolation and identification of fenobucarb degrading bacteria from Pangalengan farm land

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Abstract. The long term excessive use of pesticides can lead to their residues accumulation in the soils. Soil microbes were considered to convert the residues into harmless compounds, however the indigenous soil microbes having those beneficial properties are limited. Therefore this study aimed to isolate, select and identify the fenobucarb insecticide-degrading bacteria from agricultural soils. The soil samples were collected from the vegetable fields in Pangalengan, West Java, Indonesia. Isolation of the bacteria was conducted using Nitrate Mineral Salt Agar supplemented by 100 ppm of a fenobucarb. The bacteria isolates were selected based on its hypersensitive response, haemolytic activity, and its ability to degrade fenobucarb. The selected isolates was identified base on sequences of 16S rRNA gene. Twenty nine bacteria were isolated from four soil samples and 23 of the isolates were not potentially phytopathogenic and non haemolytic. The best three isolates that could degrade 94.2%, 94.5% and 95.47% fenobucarb residue are B41, B54 and B83 isolates, respectively. The 16S rDNA Sequence analysis showed that B41 and B83 isolates have 100% similarity to Bacillus thuringiensis MYBT 18426B54, while B54 isolate has 99% similarity to Bacillus luciferensis LMG 18422. These isolates are potential to be developed as a bioremediation agent.

1. Introduction

Farmers commonly use synthetic pesticides as one of the important components to protect their crop. Contrary to benefit of the synthetic agrochemical use, there are so many negative effect on human health and the environment [1]. Uncontrolled use of pesticides will cause adverse changes to ecosystems such as pest resistance, death of beneficial organisms, and pesticides residues in water, soil, food, and organisms [2]. One of the most widely used insecticides in the world is fenobucarb. Fenobucarb is a broad spectrum insecticides which contain BPMC (butylphenyl methylcarbamate). The insecticide has high extermination power and rapid knock down effect [3].

Naturally indigenous microbes reside in a land could degrade pesticides residue. However, the microbial capability will not proportional with the higher increasing of intensity and quantity of agrochemicals input. Efforts are needed to reduce the negative impacts caused by their use such as by implementing integrated pest control and the use of natural pesticides [4]. More over, enrichment of the polluted land by using bioremediation agent is needed. Availability of selected dan fine characterize microbial isolates is important to formulate an efective bioremediation agent. This research was conducted to isolate, screen, and indentify fenobucarb degrading bacteria from farm soil.

2. Methods
2.1. Materials
Soil samples were taken from Paddy, tomato, potato, and cabbage farmland, at Legok Bako village, Margaluyu, Pangalengan, West Java. Bacteria used in this research were isolated from the soil samples, while Ralstonia solanacearum is a collection of Biogen Culture Collection, ICABIIOGRAD.

2.2. Isolation and Purification of Fenobucarb Degrading Bacteria
Isolation and purification of the bacteria were conducted by following the procedure published by Akhdiya et. al. [5] that modified by substitute the insecticides with fenobucarb. The pure isolates were stored at -20°C in 40% glycerol as stock cultures. While as the working cultures, the isolates were streaked on NA + 100 ppm fenobucarb and stored at 15-18°C.

2.3. Hypersensitive Response (HR) Bioassay
Bacterial isolates were rejuvenated on Nutrient Agar media (Merck, US) for 24-48 hours. A colonies of bacteria were suspended in 1.5 ml of 0.85% NaCl. Half milliter of bacterial suspension was injected into the lower surface of tobacco leaf (N. tabacum L.). Ralstonia solanacearum was used as positive control. Necrotic tissue formed on the leaf were observed every day for 7 days. The experiment was conducted duplicate

2.4. Hemolytic Activity Testing
The isolates that did not cause necrotic symptoms in the previous bioassay, were inoculated on the surface of blood agar and then incubated at room temperature (25°C) for 24-72 hours. Clear zone formed around the colonies indicated haemolytic activity of the isolates.

2.5. Test to Use Fenobucarb as Exclusive C Source
The test was carried out by following the procedure published by Akhdiya et. al. [5] that modified by substitute the insecticides with fenobucarb. The cultures growth were observed visually for 24-72 hours.

2.6. Fenobucarb Degradation Test
Selected isolates were cultivated using the same procedure for use of exclusive C source. The culture were cultivated for 96 hours before its were centrifugated at 10.000 x g for 10 minutes. Supernatan of the cultures were sended to Agricultural Environmental Research Institute (IAERI) to be determined the residue. Determination of the residues was carried out using Gas Chromatography (Variant Type 450, US) equipped with VF-1701 columns and Electrone Captured Detector. The experiment was conducted in triplicate. Percentage of the degraded fenobucarb, calculated by the following equation

\[ Fd = \frac{Fi - Ff}{Fi} \times 100\% \]

Fd : Degraded fenobucarb (%)
Fi : Initial concentration of Fenobucarb
Ff : Final concentration of Fenobucarb

2.7. Identification of the Selected Isolates
DNA of the selected isolates were extracted using the PrestoTM Mini gDNA Bacteria Kit. The DNA were amplified using 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers [6]. Steps of the 30 PCR cycles were : activation (94°C, 5 minutes), denaturation (94°C, 5 minutes), annulling (55°C, 45 seconds), elongation (72°C, 1 minute), and final extension (72°C, 1 minute). Two microlitres of the amplification products were runned in
electrophoresis gel (agarosa 1%) using 1X TAE buffer at 80 volt for 40 minutes. Diamond™ Nucleic Acid Dye (Promega, US) was used to visualize the DNA bands under UV transilluminator.

The amplicon DNA were send to sequencing service laboratory. The DNA sequences were aligned with Genbank data using the BLAST-N (Basic Local Alignment Search Tool-Nucleotide) program from the NCBI (National Center for Biotechnology Information) to determine the identity of the isolates. Phylogenetic trees was created using Clustal X [7], neighbor joining method (NJ) with 1000x bootstrap.

3. Results and discussion

Fenobucarb degrading bacteria were isolated from soil samples taken from non-organic agricultural land (paddy, cabbage, tomato, and potato) in Pangalengan area, West Java. Consideration of the sampling locations was that agricultural land is managed intensively with high frequency of pesticide application. The use of NMSA + fenobucarb as the isolation medium was initial screening efforts to suppress growth of satellite colonies and to increase probability to isolate the high fenobucarb degrading capability bacteria. There were 29 fenobucarb degrading bacteria were isolated from 4 soil samples (Table 1).

| Farm land soil | Number of bacteria isolates |
|----------------|-----------------------------|
| Cabbage        | 6                           |
| Paddy          | 4                           |
| Potato         | 13                          |
| Tomato         | 6                           |

Hypersensitivity response (HR) bioassay was conducted to eliminate the phytopathogenic potential bacteria. All of the isolates tested did not cause necrotic symptom of tobacco leaves tissues (Fig. 1A and 1B). Contrary to the asymptomatic leaves which injected with the isolates, injection of R. solanacearum caused necrotic symptom of the leaf tissues. Hypersensitive response is a rapid defense reaction of plants, when facing pathogens [8]. Plant HR is indicated by formation of leaf browning tissues in the infected area due to the death of local leaf tissues [9]. The bioassay results indicated that all of the isolates are phytopathogenic.

Figure 1. Photographs of the tobacco leaves at 24 hours (A), 48 hours (B) after injected with the bacteria isolates and necrotic leaf after 48 hours after injected with R. solanacearum (C).

Next selection base on hemolytic activity of the isolates was conducted to eliminate bacterial isolates potentially pathogenic to mammals and humans. Erythrocytes lysing bacteria are potentially more virulent compared to the non haemolytic bacteria. Ability of bacteria to lysis erythrocytes is
determined by an extracellular protein “hemolisin”. Haemolysine producing bacteria will damage erythrocytes in their growth media, forming clear zones and decoloration [10]. Six of the 29 isolates were hemolytic (Figure 2).

![Figure 2](image.png)

**Figure 2.** Non-haemolytic bacteria isolates (A) and haemolytic isolates surrounded by clear zone (B)

Among the non-haemolytic isolates, 6 isolates (B41, B54, B83, B95, B96 and B97) showed grow well on liquid NMS media containing fenobucarb as sole of carbon source. Bacteria growth was indicated by increasing culture turbidity after incubation for 48 hours. The turbidity of liquid culture in NMS containing pesticide is caused by the growth of bacteria that utilize pesticides as sole of carbon source for their growth [11]. Fenobucarb resistant isolates but can not use the compound as its C and energy source can not grow in the medium. Carbon is one of the main element of cells macro molecules. There are four organic macro molecules: proteins, nucleic acids, carbohydrates and fats that have different chemical properties [12]. All organisms needs this element in relatively large quantities for its growth.

| Isolate code | Degraded pesticides (%) |
|--------------|-------------------------|
| B41          | 94.27                   |
| B54          | 94.57                   |
| B83          | 95.29                   |
| B95          | 75.47                   |
| B96          | 69.65                   |
| B97          | 67.19                   |

*Table 2.† Fenobucarb residue in liquid culture 6 isolates selected after 96 hours incubation*

Analysis of fenobucarb residues in the culture of 6 selected isolates showed its ability to degrade the compound were 67.19% - 95.29% within 96 hours. Isolates B83, B54, and B41 could degrade respectively 95.29%, 94.57% and 94.27% fenobucarb residue (Table 2). Fenobucarb is an aromatic hydrocarbon compound. Ability of microorganisms to degrade aromatic hydrocarbon compounds are varies. The differences can be caused by the type, quantity, and activity of pesticide-degrading enzymes producing by the organisms. Bacterial enzymes that play a role in degradation process of aromatic hydrocarbon compounds include dioxygenases, monooxygenases [13], and hydrolytic enzymes [14, 15]. Monoxygenase and dioxygenase enzymes are able to open carbon bonds of the aromatic rings and produce primary alcohol by using oxygen molecules as electron acceptors. The process will produces dicarboxylic and semi-aldehyde acids that are useful for the metabolism of cell intermediates [15]. Decreasing of fenobucarb concentration in the cultivation media after incubation is the result of bacteria metabolism. Major chemical bonds in the toxic molecules can disrupt by hydrolytic enzymes and results in the reduction of its toxicity. This enzymatic mechanism is
effective for the biodegradation of hydrocarbon compounds include oil, organophosphate, and carbamate [15]. Fenobucarb is degraded through a hydrolysis reaction by producing the main product 2-sec-buthylphenol. The hydrolysis product, then metabolized into carbon dioxide and water [16]. Many publications reported that Bacillus spp. Have dioxygenase [17], hydroxylase [18], and hydrolases [15, 19, 20]. Phosphatase and esterase are hydrolytic enzymes produced by soil microbes that can break the unstable chain structure such as carbamate [21].

Amplification of 16S rDNA sequences of the three selected isolates using primers 27F and 1492R produced ± 1,500 bp DNA fragments. Molecular identification results based on 16S rDNA sequence analysis showed that B54 isolate has 99% similarity Bacillus luciferensis LMG 18422 (99% homology), while B41 and B83 isolates show the highest similarity (99%) with Bacillus thuringiensis MYBT 18246. The phylogenetic tree constructed base on the sequences showed the closely related Bacillus species with Bacillus luciferensis B54 and Bacillus thuringiensis B41 and B83 (Figure 3).

**Figure 3.** Phylogenetic tree of three phenobucarb-degrading Bacillus isolates based on its 16S rRNA sequences.

4. **Conclusion**
B. thuringiensis B41, B. thuringiensis B83, and Bacillus luciferensis B54 have high capability to degrade fenobucarb. The Bacillus were potential to be develop as bioremediation agents. Further testing is needed to determine its ability to degrade other pesticides

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