Growth ability and denitrification activity of bacterial isolates on media containing propoxur

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Abstract. Denitrifying bacteria are expected to be a promising organism to degrade pesticides by using them as carbon and nitrogen substrate. The potential of denitrifying bacterial isolates namely TK Bali, KT, UHT, L7T4, and C.pkr in degrading propoxur was studied through growth ability test and denitrification activity on three types of media containing propoxur. The media were NB (Nutrient Broth), MS (Mineral Salt), and MS + glucose added with various concentrations of propoxur (500, 1000, 1500 ppm). Bacterial growth was analyzed by measuring turbidity (OD) at a wavelength of 436 nm. Denitrification activity was measured by nitrate reduction capacity. Concentration of propoxur and isopropoxyphenol were measured using HPLC. The results showed that the highest growth and denitrification activities were in NB media. Denitrifying bacteria can grow in NB media containing propoxur up to 1500 ppm concentration, but the growth and denitrification activities decreased with the increasing concentration of propoxur. The denitrifying bacteria isolates seemed not to be able to use propoxur as the sole source of carbon and nitrogen. Denitrifying bacteria were resistant to propoxur to a certain concentration, but were unable to degrade propoxur, or it could degrade propoxur, but only in very low capacity. Isolate Brevundimonas diminuta, L7T4 was able to degrade propoxur to isopropoxyphenol in relatively low rate of degradation, less than 20 % of propoxur was degraded in 2 days of incubation.

1. Introduction
One type of carbamate pesticide that is currently widely used is propoxur. Propoxur is a phenyl N-methylcarbamates pesticide comprising 2-isopropoxyphenol N-methylcarbamate [1]. Propoxur is a broad spectrum insecticide; used in hospitals, factories, houses and stables at a concentration of above 0.5% active matter for control ling flies, ants, aphids, mosquitoes, cockroaches and millipedes [2]. Propoxur has the potential to inhibit cholinesterase and is toxic in humans as well as animals with LD50 of 100 mg / kg [3]. Propoxur has an effect on various soil microbes, among others, against nitrifying bacteria. Propoxur is able to inhibit the growth of some nitrifying bacteria, inhibition can occur for 2-3 weeks after the use of propoxur on soil with a concentration of 500 ppm [4]. The use of pesticides is often inappropriately targeted causing environmental problems because it has an effect on non-target microorganisms, ie reducing the population, enzyme activity, species diversity, and changes in microbial community structure [5]. Propoxur is readily degraded by soil microorganisms in most soils. Environmental conditions that favor the growth and activity of microorganisms also favor degradation. Hydrolysis is a major degradation pathway in soil. Propoxur is reported to be biodegraded quite rapidly in water, particularly when the bacterial activity and temperature is high. 2-Isopropoxyphenol
is a product of propoxur biodegradation [2,6,7,8]. Bacteria capable of degrading carbamate pesticides have been isolated from the soil [2,6,8,9]. Studies have implicated the involvement of Pseudomonas species in the degradation of propoxur [3]. Many studies have indicated that the first step in the microbial degradation of carbamate compounds is the hydrolysis of the carbamate linkage [7,9,10,11].

Excessive nitrogen compounds in soils resulting from the application of chemical fertilizers are also serious environmental problem. Nitrate leaching is the effect of excess of nitrogen compounds in agricultural areas that often lead to contamination of surface water. Denitrification plays an important role in the removal of nitrate (NO$_3^-$) rather than through assimilation by plants and leaching. Denitrification is a process of bacterial respiration in the process of reducing nitrate (NO$_3^-$) to N$_2$O, NO, and N$_2$. Nitrite or nitrate ions will be used as the last electron recipient in nitrate respiration or nitrate dissimilation [12]. Denitrifying bacteria are heterotrophic bacteria, they require organic carbon sources for their growth. Various sources of organic carbon can be used as electron donor [13]. Denitrifying bacteria is expected to be a promising tool in degrading nitrates and pesticides simultaneously from an environment. Furthermore, denitrifying bacteria degraded pesticides by using them as carbon and nitrogen substrate. The research report on anaerobic pesticide degradation using denitrification bacteria is still very limited. Therefore it is important to explore the potential of denitrifying bacteria in the degradation of propoxur by testing the growing ability and activity of denitrifying bacteria on media containing propoxur.

2. Materials and Methods

2.1. The qualitative test of growth and denitrification activity

The growth and denitrification activity of five bacterial isolates (TK Bali, KT, UHT, L7T4, C.pkr) were tested qualitatively in the test tube containing Giltay and Nutrient Broth (NB) media with 500 ppm propoxur. Each bacterial isolate was grown in 5 test tubes. The test tube was blocked with 3 ml of liquid paraffin which created anaerobic condition. Bacterial culture was then incubated for 14 days at room temperature. Indications of the growth of denitrification bacteria are characterized by the formation of gas in the durham tube and the blue color change from Giltay media.

2.2. The growth and denitrification activity test of bacteria on 3 types of media containing 1000 ppm propoxur

Five bacterial isolates (TK Bali, KT, UHT, L7T4, C.pkr) were grown on a test tube containing liquid medium of NB + 500 ppm KNO$_3$. The bacterial culture was incubated for 24 hours at room temperature. Each bacterial isolate was grown in 3 types of media, by inoculating 1 ml bacterial culture on glass bottle containing 3 different media ie NB, Mineral Salt and Mineral Salt+glucose. All three media were added with 1000 ppm propoxur. Bacterial culture was incubated in thermost waterbath for 3 days. Each culture was sampled 3 ml every day (24 hours) and stored in eppendorf to measure OD and nitrate analysis (NO$_3^-$). OD measurements are carried out using a UV-VIZ spectrophotometer (UV mini 1240 SHIMADZU) with a wavelength of 436 nm. The sample used for NO$_3^-$ analysis was centrifuged for 10 minutes at a speed of 12,000 rpm. The supernatant was then used for NO$_3^-$ analysis.

Nitrate reduction capacity : \(
\frac{(NO_3)_t-(NO_3)_0}{(NO_3)_0} \times 100 \%
\)  

\(0 = \) 0 day  
\(t = 3 \) days
2.3. The growth and denitrification activity test of bacteria on NB media with various concentration of propoxur
Five bacterial isolates (TK Bali, KT, UHT, L7T4, C.pkr) were tested for their growth ability and denitrification activity on media supplemented with various propoxur concentrations. The bacterial isolates that will be tested were grown in test tubes containing liquid medium of NB + 500 ppm KNO₃. The bacterial culture was incubated for 24 hours at room temperature. Each bacterial isolate was taken as much as 1 ml and grown in a glass bottle containing 500 ml of NB + KNO₃ with various concentrations of propoxur (500 ppm, 1000 ppm, and 1500 ppm). The bacterial culture was then incubated in a thermo waterbath for 48 hours and 3 ml of the culture was sampled by using tubes every 24 hours for OD measurement and nitrate analysis (NO₃⁻).

Nitrate reduction capacity : \[
\frac{(NO_3)_0 - (NO_3)_t}{(NO_3)_0} \times 100 \% \tag{2}
\]

0 = 0 day
\(t = 2\) days

2.4. The growth and denitrification activity (nitrate reduction), and propoxur degradation (propoxur reduction) of selected bacterial isolate
One selected bacterial isolate (L7T4) which growth stable on media containing various concentration of propoxur was grown in a test tube containing liquid medium of NB + 500 ppm KNO₃ and incubated for 24 hours at room temperature. The culture of this bacterial isolate was taken 2.5 ml and put in a schott bottle containing 250 ml of NB + 500 ppm KNO₃ and 1000 ppm of propoxur. Bottle culture is conditioned to be anaerobic by flushing Argon gas for approximately 25 minutes. This bacterial culture was incubated with stirring for 48 hours and sampling as much as 3 ml by using tubes every 4 hours for growth measurement (OD), and nitrate (NO₃⁻), and propoxur analysis.

2.5. Analyses of NO₃⁻
A total of 3.6 ml of pH 7 distilled water was mixed with 0.4 ml of sample (10 x dilution) and put into a large test tube. In a tube, 0.8 ml of 30% NaCl solution was added, and then 4 ml of 75% sulfuric acid (H₂SO₄) solution was added. Each addition of the solution is shaken using a vortex to be perfectly mixed. The mixture is cooled down first and then added with 0.2 ml of a mixture of brucine and sulfuric acid. The tube is then heated in a waterbath at 95 °C or put into a pan containing boiled water at a temperature of 95°C for 20 minutes. Nitrate (NO₃⁻) concentration is measured by UV-VIS spectrophotometer (UV mini 1240 SHIMADZU) at a wavelength (λ) 410 nm [14]. The color obtained will be used for data analysis using standard nitrate curves that have been made previously.

2.6. Propoxur degradation assay
The concentration of propoxur and 2-Isopropoxyphenol in the culture supernatant was analyzed by HPLC (SHIMADZU Prominence LC-20A). HPLC chromatograms were produced by injecting 20 µL of the supernatant onto a 5 µm reverse phase column (Li Chrosphere 100 RP-18 end capped) and recorded by ultraviolet detector at 280 nm. The mobile phase was acetonitrile : water (60:40 (v/v), flow rate was 1 mL/min. The retention time of peaks were compared to those of chemical compound authentic standards. Analytical standards for 2-Isopropoxyphenol was purchased from Sigma Chemical Co. Propoxur Tech was gift from PT Inti Everspring Indonesia. Methylamine was obtained from Merck. All other chemicals were analytical grade and purchased internationally and locally.

2.7. Identification of L7T4 isolate
Isolate L7T4 was identified based on 16S rDNA analyses. DNA was extracted and amplified by direct colony PCR. Amplification product was then sent to 1st BASE for sequencing analyses. Related sequences were obtained from GenBank database of National Center for Biotechnology Information (NCBI), using BLAST. The sequences were aligned and the consensus sequences was computed using
MUSCLE program. Phylogenetic tree for the data sets were inferred by the neighbor-joining method by using the neighbor-joining program, MEGA version 7.

3. Results and Discussions

3.1. Qualitative test of growth and denitrification activity
Five bacteria isolates of L7T4, UHT, Tk Bali, KT, CPkr showed indications of being able to grow and carry out denitrification reactions, indicated by the formation of gas in durham tubes on Giltay and NB media, both in control and treatment (Table 1). In addition, the five bacterial isolates were able to change the color of the Giltay media from green to blue, indicating a change in pH in the media into bases. In general, denitrification can occur well at optimum pH between 7-8 [15]. Denitrification activity is characterized by the formation of N₂O and N₂ gases [16]. Denitrification activity is also characterized by changes in Giltay media color from green to blue, the color change is caused by changes in pH to bases due to the formation of OH⁻ in denitrification reactions. Addition of 500 ppm propoxur concentration did not inhibit growth and denitrification activity of the five bacterial isolates tested.

Table 1. The qualitative test results of growth and denitrification activity

| Isolate Code | Giltay Media (500 ppm propoxur) | NB Media (500 ppm propoxur) | Giltay Media (Control) | NB Media (Control) |
|-------------|---------------------------------|-----------------------------|------------------------|-------------------|
|             | Gas Color                        | Gas Color                   | Gas Color              |                   |
| L7T4        | + Blue                           | + yellowish green           | +                      |                   |
|             |                                 | + Blue                      | + yellowish green      |                   |
| UHT         | + Blue                           | +                           | + Blue                 |                   |
|             | + Blue                           | +                           | + Blue                 |                   |
| Tk Bali     | + bluish green                  | +                           | + bluish green         |                   |
|             |                                 | + bluish green              | + green                |                   |
| KT          | + Blue                           | +                           | + green                |                   |
|             | + Blue                           | +                           | + green                |                   |
| CPkr        | + Blue                           | +                           | + blue                 |                   |
|             | + bluish green                  | +                           | + blue                 |                   |

3.2. The growth and denitrification activity test of bacteria on 3 types of media containing 1000 ppm propoxur
All bacterial isolates grew well on NB media, while on both two media MS and MS + glucose, grew very slowly (Figure 1). The results of measurements of nitrate reduction activity for 6 days of incubation on NB media were the fastest (90-98%), followed by MS media (50-60%) and MS + glucose media (30-40%). This slowing reduction of NO₃⁻ was related to the bacterial growth which was also slow (Figure 2). Decrease in nitrate is an early indication of denitrification reaction, where there is a reduction of nitrate (NO₃⁻) to N₂O, NO, and N₂ [12].
Denitrification bacteria are heterotrophic bacteria, they need organic carbon for growth and activity. The type of organic carbon needed varies greatly depending on the type of bacteria. If the availability of low organic material will cause low growth, the denitrification activity will also decrease. From the results of this test, it can be suspected that the five bacterial isolates tested cannot use propoxur as the only carbon source. This was evident from the very slow growth of bacteria at mineral salt media which was supplemented with propoxur alone. Growth and nitrate reduction in MS + glucose media was also slow, this indicated that glucose is not the right organic carbon compound for the five isolates tested. Whereas on the NB media, denitrification bacterial isolates grew well and nitrate reduction activity was also high because it contained a good carbon source. A wide variety of organic compounds has been used, such as methanol, ethanol, glucose, acetate, aspartate or formic acid and aromatic compounds [17]. However, most of the published research regarding drinking water denitrification involved the use of methanol, ethanol and acetic acid [18].

**Figure 1.** Growth of five bacterial isolates on 3 types media.

**Figure 2.** Nitrate reduction capacity of 5 bacterial isolates on 3 types of media containing 1000 ppm propoxur.
3.3. The growth, denitrification activity and degradation of propoxur in NB media by giving various concentration of propoxur

The results showed that the highest growth and denitrification activities were in NB media. Denitrifying bacteria can grow in NB media containing propoxur up to 1500 ppm concentration, but the growth and denitrification activities decreased with the increasing concentration of propoxur (Figure 3). This showed that propoxur inhibits the growth of denitrification bacteria, the same thing also happened with nitrifying bacteria [4]. Bacterial isolates L7T4 showed relatively best growth in almost all propoxur concentrations compared to the other bacterial isolates.

![Figure 3](image-url)  
**Figure 3.** Growth of five bacterial isolates on NB media with various concentrations of propoxur.

On the Figure 4, it was shown that there was a process of nitrate reduction in the five tested bacterial isolates, a decrease in nitrate was an early indication of the occurrence of the denitrification process. In general, the decrease in nitrate that occurred was quite good, reaching 100% in the media without propoxur, and slightly decreased (80-90%) with the increase in propoxur content. Even the UHT bacterial isolate was able to reduce nitrate concentrations to almost 100% in the concentrations of propoxur 500, 1000 and 1500 ppm. This result was different from the result of growth which was experiencing inhibition (Figure3). This showed that propoxur compounds have more influence on bacterial growth compared to nitrate reduction (denitrification) activity.

![Figure 4](image-url)  
**Figure 4.** Nitrate reduction capacity of 5 bacterial isolates on NB media containing various concentrations of propoxur.
Figure 5. Formation of isopropoxyphenol in various bacterial isolates and propoxur concentrations.

The formation of isopropoxyphenol is an indication of the degradation of propoxur, because isopropoxyphenol is one of the degradation results of propoxur. Formation of 2-Isopropoxyphenol appeared to increase along with the increase of propoxur administration. However, the formation of 2-Isopropoxyphenol was very small if compared with the concentration of propoxur in the media. For example, the L7T4 isolate treatment, in the media with 1000 ppm of propoxur, only about 70 ppm of isopropoxyphenol were formed, it means that the degradation of propoxur was only about 7%.

3.4. The growth and denitrification activity (nitrate reduction), and propoxur degradation (propoxur reduction) of selected bacterial isolate

One denitrification bacterial isolate (L7T4) was then further tested for denitrification activity and the ability to degrade propoxur. The growth of L7T4 bacterial isolates on NB media containing 1500 ppm of propoxur was quite good (Figure 6A). The growth curve of L7T4 bacterial isolates has 3 phases, namely the lag phase at 0 to 8 hours, the exponential phase on the 8th to 24th hours, and the stationary phase at the 24th to 48th hours. However its growth was relatively slow, within 48 hours it only reached 1.6 (OD). These bacterial isolates were also able to carry out denitrification activity which was indicated by the decrease in nitrate (NO$_3$-N) concentration from 33.42 ppm to 0 (100%) during 48 hours incubation (Figure 6B). However, propoxur degradation activities by isolate L7T4 was very low, only about 20 % during two days incubation (Figure 6C)

From this study it was shown that the five denitrification bacteria were able to grow and carry out denitrification activities on NB media containing propoxur to a concentration of 1500 ppm. This means that denitrification bacteria were resistant to propoxur to a certain concentration, but were unable to degrade propoxur, or it could degrade propoxur, but only in very low capacity. This fact showed that agrochemical ingredients (propoxur) inhibit the growth and activity of microorganisms in the soil, such as denitrifying bacteria. However, some bacteria are able to survive or are resistant to agrochemical compounds to some extent.
Figure 6. Growth curve, nitrate and propoxur reduction activity of L7T4 isolates.

Figure 7. Phylogenetic tree base on partial sequent 16S-rRNA gene.
Blast result showed that L7T4 isolate is 99% similar to genus Brevundimonas. From Figure 7 we can see the relationship of L7T4 to members of genus Brevundimonas and closes related genera. From the phylogenetic tree it was shown that L7T4 was closed to *Brevundimonas diminuta*. The genus *Brevundimonas* was proposed based on the reclassification of two Pseudomonas species, *Pseudomonas diminuta* and *Pseudomonas vesicularis*, as *Brevundimonas diminuta* and *Brevundimonas vesicularis* [19,20,21]. One strain of this genus is *Brevundimonas denitrificans* sp. nov. This bacterium is denitrifying bacteria that can reduce nitrate. These bacteria are also very sensitive to various chemicals, such as bacitracin and novobiocin, weakly sensitive to cefmetazole, cefoxitin, kanamycin, neomycin etc. Identifiable growth is observed on D-mannose, D-mannitol, N-acetyl-D-glucosamine, potassium gluconate and sodium citrate but not on glucose (API 20NE) [22].

4. Conclusion
The denitrifying bacteria isolates seemed not to be able to use propoxur as the sole source of carbon and nitrogen. Denitrifying bacteria were resistant to propoxur to a certain concentration, but were unable to degrade propoxur, or it could degrade propoxur, but only in very low capacity. Agrochemical ingredients (propoxur) inhibit the growth and activity of microorganisms in the soil, such as denitrifying bacteria. Isolate *Brevundimonas diminuta*, L7T4 was able to degrade propoxur to isopropoxyphenol in relatively low rate of degradation, less than 20 % of propoxur was degraded in 2 days of incubation.

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Acknowledgements
We are very grateful to technicians of their valuable assistants to finish this study. This work was supported by DIPA project, Indonesian Institute of Sciences.