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Isolation and Characterization of Buprofezin Tolerant Bacteria from Rhizosfer of Paddy at Marginal Land of Banyumas Regency

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Abstract. Buprofezin is a pesticide used to control planthoppers. The buprofezin residue may reduce the soil fertility by decreasing the essential microbes in the soil. This study aimed to isolate and characterizes buprofezin tolerance bacteria from rhizosfer of paddy at five marginal land of Srowot, Pageralang, Gunung Tugel, Tamansari, and Sokawera village, Banyumas Regency, Central Java. The bacterial isolate screened by growing on nutrient agar containing 2 ppm of buprofezin. The growing colonies were macro-morphologically observed. The growth curve and the generation time of the dominant colonies were analyzed. The selected colonies were cultured in nutrient broth containing 0, 5, 10, and 15 ppm of buprofezin. The selected colonies were characterized by gram staining, endospore staining, and biochemical test. Thirty collected-bacterial isolates showed five dominant colonies (SR1, PA1, GT2, TS4, SW1). The selected dominant isolates, SR1 colony was tolerant to buprofezin at 5 ppm and 10 ppm and GT2 at 5 ppm of buprofezin. The SR1 and GT2 were rod-shaped, gram-positive and endospore forming bacteria, white, and medium in size. The biochemical tests showed the SR1 were motile, had catalase activity, can ferment glucose, sucrose, and mannitol without gas forming, unable to ferment lactose, hydrolyze starch, positive result for MR test, but negative for urease, VP test, simmons citrate, H_2S production, oxidase, and indole. The GT2 were motile, positive result for catalase test, carbohydrate fermentation (glucose, sucrose, mannitol, lactose) with gas forming, MR/VP test, simmons citrate, oxidase, and indole, but negative for starch hydrolysis, urease, and H_2S production. The SR1 and GT2 were aerobic/anaerobic facultative bacteria. SR1 and GT2 were probably *Bacillus* sp.

Keyword: Bacteria, Marginal land area, Banyumas, *Buprofezin,* Biochemical-test

1. Introduction

Buprofezin is one active pesticide material used in pest management of planthoppers [11]. Meanwhile, the value of DT50 (half-life) of pesticides with the active *buprofezin* material in soil under aerobic conditions is 26-220 days [15]. The *buprofezin* residue which is long deposited in the soil may potentially reduce its fertility level. The *buprofezin* residue may decrease the activity of *urease*, *protease*, and *phosphatase* in the soil [15]. Soil bacteria are known to produce *urease* [2][3][12][13], *protease* [8][17] and phosphatase [4]. Some soil bacteria are known to be tolerant with soil conditions containing *buprofezin* residue. Even groups of bacteria, such as *Rhodococcus sp*. are known to be able to reduce buprofezin residue [9][10].

One potential source to obtain bacteria tolerant to *buprofezin* is the soil from the marginal land areas. Marginal land areas in Indonesia are known to have more than 100 million hectares [18]. One region in Indonesia which has some marginal land areas is Banyumas Regency, Central Java. Some marginal land areas in Banyumas Regency have been used for rice cultivation activities. Rice that is cultivated is generally upland rice, which has dry environmental resistance characteristics. Knowledge of indigenous marginal bacteria around rice roots and their tolerance to buprofezin compounds opens opportunities for bacterial development in an effort to reduce the level of buprofezin residue contamination in agricultural areas, especially on marginal lands.

This study aimed to isolate and characterizes buprofezin tolerance bacteria from rhizosfer of paddy at five marginal land of Srowot, Pageralang, Gunung Tugel, Tamansari, and Sokawera village, Banyumas Regency, Central Java.

2. Materials and Methods

2.1 Soil sampling Collection

The soil samples were collected with a composite method. For each location, 20 grams of the soil sample was taken from a depth of 20 cm from the soil surface around the roots of rice plants. The soil was homogenized and then stored in a sterile plastic and placed in a 4^{O}C sample box [5].

2.2 Isolation of Soil Bacteria

The bacteria were isolated by a serial dilution method (from 10^{-1} to 10^{-8}) and spread on Nutrient Agar (NA) (Merck KGaA) media which contain 2 ppm *buprofezin*, incubated at 30^{O}C for 48 hours. The growing colonies were calculated with a total plate count (TPC) or a total population in colony forming units (cfu/gram) and grouped based on its morphological observation. The grouped colonies were then calculated in order to obtain the dominant colonies in each sampling area. Dominant bacterial colonies were cultured on NA media to obtain single isolate for gram staining and growth rate analysis to determine the regeneration time.

2.3 Dominant colonies'growth rate

The bacterial growth rate was obtained from the measuring results of optical density (OD) or absorbance at the wavelength of 600 nm within Nutrient Broth (NB) (Merck KGaA) media using a spectrophotometer (UVD 3200, Labomed.inc). OD measurement is conducted every two hours. From the obtained growth rate curve, the time generation of each dominant colony may be calculated. The dominant colony with the most appropriate growth rate and the fastest generation time will undertake a tolerance test towards pesticide with active *buprofezin* material on various concentrations.

2.4 Tolerance test on media containing buprofezin

A tolerance test was conducted on the selected dominant colonies of bacteria on NB media which contain *buprofezin* 0, 5, 10, and 15 ppm. The tests were conducted for 72 hours at the temperature of 30^{O}C and speed of 150 rpm. The bacterial tolerance may be figured out from the growth rate curve by measuring the media turbidity level with a spectrophotometric method at χ 600 nm.

2.5 Characterization of selected isolates by gram staining

The colony of bacteria spreaded on an already cleaned object glass with alcohol 70%. The colony then fixed by heating the object glass. After cooling, 2-3 drops of crystal violet (Merck) solution added on the object glass and let it stand for 1 minute. The object glass cleaned with alcohol 70% and then distilled water (C'Jaya QMia) added and dried. Next, the *Lugol* (Merck) solution dropped into the object glass and let it stand for 1 minute, the washed with the running distilled water and dried. To the object glass added acid alcohol solution for 30 seconds, washed with water, and then dried. Safranin (Merck) solution then added on the object glass and let it stand for 20 seconds. The object glass cleaned with the running water and then dried. The stained bacterial isolate observed under a light microscope with a magnification of 1000 times.

2.6 Characterization of selected isolates by endospore staining

Endospore staining test based on Schaeffer-Fulton method. Malachite Green (Merck) dyes was added to the smear of bacterial cells on the object glass until submerged. Bacterial cells were fixed by heating for 10 minutes (every 1 minute added 1 drop of malachite green). The samples then cooled and washed with flowing distilled water. Safranin counter stain was added to the mixture and incubated for 1 minute. The samples were washed with flowing distilled water and air dried. The sample were added immerse oil (Merck) and observed by light microscope with 1000x magnification.

2.7 Characterization of selected isolates by biochemical testing

Selected buprofezin-tolerant bacteria isolate were characterized through a series of biochemical tests such as motility, catalase activity, sugar fermentation ability (glucose, sucrose, mannitol, and lactose), hydrolysis of starch, methyl-red (MR) and voges-prostekuer (VP), simmons citrate, H_2S production, oxidase activity, indole production, and bacterial metabolism in the media containing liquid paraffin (paraffin test).

3. Result and Discussion

3.1 Number of bacterial populations

The average total plate count (cfu/gram) of colonies in each sampling location was presented in Table 1. The largest bacterial population was obtained from the sample taken in Sokawera village by $1.2x10^{10}$cfu/gram, while the lowest one was from that taken in Pageralang village by $2.0x10^{7}$cfu/gram.

Table 1. Average total plate count

Sampling Location	Isolate code	Average TPC (cfu/gram)
Srowot Village	SR	2.5×10^{9}
Pageralang Village	PA	2.0×10^{7}
GunungTugel Village	GT	7.5×10^{8}
Tamansari Village	TS	2.4×10^{8}
Sokawera Village	SW	1.2×10^{10}

The observation results of general morphology based on the configuration of colony color, edge border, and size showed that there were 30 bacterial colonies (Table 2). The colony configuration mostly has a round shape, white color, and medium size, while the colony edge border tends to be various. The further grouping results showed that there were five dominant bacterial colonies based on the largest of population: SR1, PA1, GT2, TS4, and SW1.

Table 2. The observation results of colonies'general morphology

No	Isolate Code	Configuration	Color	Edge Border	Size
1	SR1	Round	White	Smooth Transparent Margin	Medium
2	SR2	Round	Clear	Smooth	Medium
3	SR3	Spreading	White	Irregular	Big
4	SR4	Round	White	Lobate	Medium
5	PA1	Round	White	Smooth	Small
6	PA2	Round	White	Lobate	Big
7	PA3	Round	White	Irregular	Medium
8	PA4	Round	White	Point	Small
9	PA5	Round	White	Winked Smooth	Medium
10	GT1	Fitform	White	Semi Transparent Margin	Medium
11	GT2	Round	White	Smooth Raised Center	Medium
12	GT3	Round	White	Irregular Raised Center	Medium
13	GT4	Round	White	Radating	Medium
14	GT5	Round	White	Caliate	Medium
15	GT6	Round	White	Smooth	Medium
16	GT7	Round	White	Smooth	Medium

17	GT8	Round	White	Rhizoid	Medium
18	GT9	Filamentous	White	-	Medium
19	GT10	Winked Center	White	Irregular	Medium
20	TS1	Round	White	Smooth Raised Center	Small
21	TS2	Round	White	Smooth	Medium
22	TS3	Round	White	Smooth Flat	Medium
23	TS4	Round	White	Point Center	Medium
24	TS5	Round	White	Lobate	Big
25	SW1	Round	White	Smooth	Small
26	SW2	Round	White	Transparent Margin	Medium
27	SW3	Filamentous	White	Lobate	Medium
28	SW4	Complex	White	Irregular	Medium
29	SW5	Round	White	Undulate	Small
30	SW6	Filamentous	White	Irregular	Medium

Remarks: Dominant colonies based on the largest population were marked in black background

3.2 Dominant colonies growth rate

After 28-hour observation on the growth rate of five dominant colonies, the results showed that colony SR1 and GT2 have the growth curve similar to the common bacterial growth curve, while the others have the growth curve with fluctuating rate (growth rate curve was not shown). Based on generation time, the colony SR1 and GT2 respectively have the generation time of 40 and 56 minutes, while the colony PA1, TS4, and SW1 respectively have the generation time of 1 hour 31 minutes, 2 hours 13 minutes, and 48 minutes. Based on the growth curve combined with the generation time, the colony SR1 and GT2 were selected to be examined with the tolerance test to pesticides with *buprofezin* at various concentrations.

3.3 Tolerance test conducted on bacteria towards buprofezin

The selected colony isolates, SR1 and GT2, were examined for their tolerance on NB media which contain pesticides with *buprofezin* 0, 5, 10, and 15 ppm. The observation results on colony growth rate of SR1 and GT2 on NB media which contain various concentrations of pesticides with *buprofezin* were presented in Figure 2 and Figure 3.

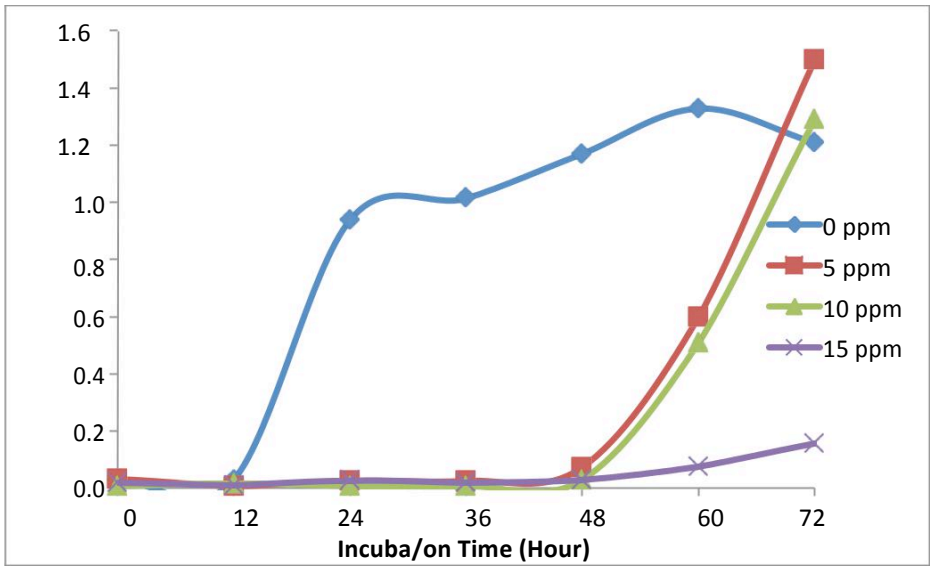

Figure 1. The growth curve of SR1 concentrations of *buprofezin on* NB

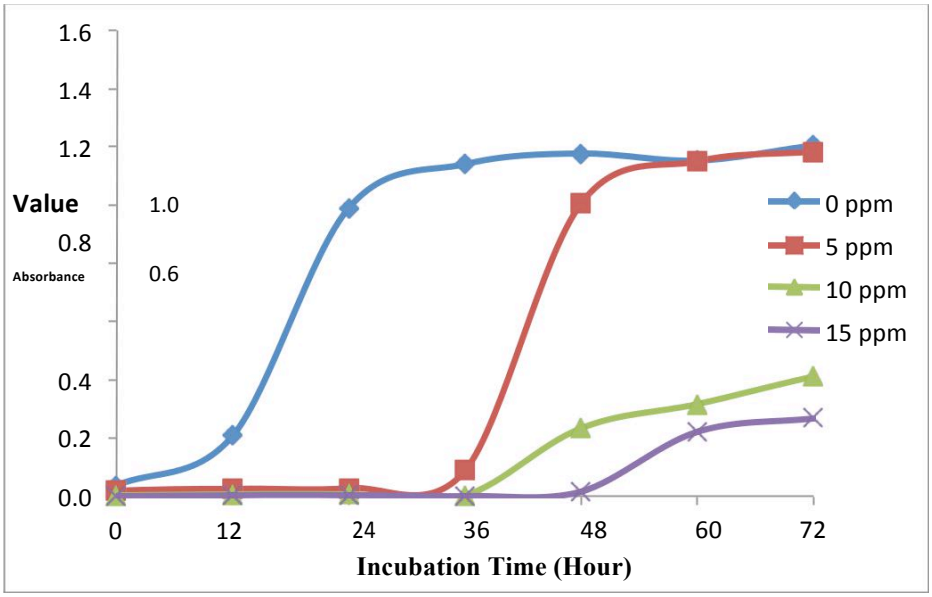

Figure 2. The growth curve of GT2 isolates on various concentrations of *buprofezin* on NB

Based on Figure 1, the SR1 bacterial isolates growth appears to be suppressed due to the presence of *buprofezin* with the concentration of 5, 10, and 15 ppm. The growth curve was shown gradually sloping up to the 48th hour while the control one was only sloping up to the 12h. It is similar to that shown by the bacterial isolate GT2. The presence of *buprofezin* 5, 10, and 15 ppm suppressed the growth of bacterial isolate GT2. The growth curve was shown gradually sloping up to the 36h, when compared to the control one. It shows that the presence of *buprofezin* in the media makes the bacterial lag or adaptation phase becomes longer. According to [5], bacterial growth rate on media containing pesticides seems linear during incubation for several days. It is assumed that bacteria have

not optimally utilized the available nutrients in the media. The presence of pesticides makes the bacteria experience stress and inhibits the bacterial growth during the adaptation process [7].

After 48h the growth rate of SR1 bacterial isolates on media which contain *buprofezin* 5 and 10 ppm drastically increase, while at 15 ppm concentration the growth rate tends to slope gradually. The exponential growth phase of SR1 isolates at the concentration of 5 and 10 ppm was shown higher than the control one. In this condition, it is assumed that the bacteria optimally start utilizing the nutrients available on media as well as the *buprofezin* as an additional nutrient source. Bacterial isolates may use pesticides as a carbon source for their growth [14][19].

It is slightly different when GT2 colony isolates were compared with the SR1 isolates that the exponential growth rate increase after 36 minutes on media with *buprofezin* concentration of 5 and 10 ppm, while in media with the concentration of 15 ppm increases after 48 minutes. However, the increasing rate was not greater than that of the control. In facts, the exponential growth rate of isolates at *buprofezin* concentration of 10 and 15 ppm was even much lower than that of the control. In this condition, it is assumed that bacteria start optimally utilizing the nutrients available in media, yet not utilizing *buprofezin* as an additional nutrient source.

According to the tolerance observation results on media containing *buprofezin* based on the bacterial growth curve, the SR1 isolates may well tolerant to *buprofezin* concentration of 5 and 10 ppm, while GT2 isolates may only tolerant of 5 ppm. SR1 Isolates require a longer adaptation time, that is, 48 hours when compared to that of GT2 isolates by only 36 hours.

3.4 Gram and endospores characteristic of selected isolate

Gram and endospores staining and shape observation were further conducted to SR1 and GT2 selected bacterial colonies under a light microscope with the magnification of 1000 times. The results of gram and endospore staining conducted on those selected bacterial colonies were presented in Table 4. All colonies were identified as gram-positive and endospore forming bacteria. The results of gram staining and microscopic observations conducted on the appearance of those colonies were presented in Figure 3.

Table 3. Gram staining result of selected bacterial colonies

No	Isolate	Gram Staining	Endospores Staining	Colony Shape
1	SR1	Positive	Positive	Rod
2	GT2	Positive	Positive	Rod

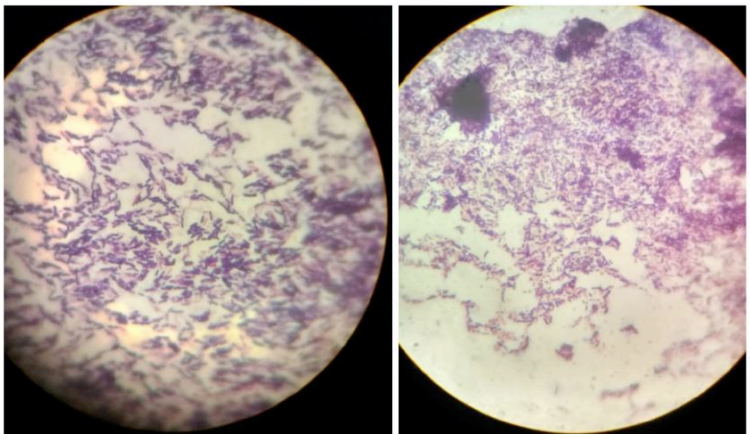

Figure 3. Gram staining results of SR1 (left) and GT2 (Right). The colonies showed that those rod-shaped gram-positive bacteria

3.5 Biochemical characteristic of selected isolate

The biochemical test results of selected isolates of SR1 and GT2 bacteria were presented in Table 4.

Table 4. Biochemical Test Result of SR1 and GT2

Biochemical Test	Isolate	
	SR1	GT2
Motility Test	+	+
Catalase Test	+	+
Glucose Fermentation Test	Acid, without gas	Acid, with gas
Sucrose Fermentation Test	Acid, without gas	Acid, with gas
Mannitol Fermentation Test	Acid, without gas	Acid, with gas
Lactose Fermentation Test	-	Acid, with gas
Starch Hydrolysis Test	+	-
Urease Test	-	-
MR Test	+	+
VP Test	-	+
Simmons Citrate Test	-	+
H_2S Production Test	-	-
Oxidase Test	-	+
Indole Test	-	++
Paraffin Test	Facultative Aerobes/Anaerobes	Facultative Aerobes/Anaerobes

Based on the results of the biochemical tests presented in Table 4, the SR1 and GT2 isolates have similar test results, which are positive for motility, catalase, MR, paraffin and negative for urease and H_2S production tests. The two isolates showed different characteristics in the sugar fermentation test, starch hydrolysis, VP, simmons citrate, oxidase, and indole. Isolate SR1 showed positive results for glucose, sucrose and mannitol fermentation. The media turns acidic but no gas bubbles form. Meanwhile, GT2 isolates showed positive for glucose, sucrose and mannitol fermentation characterized by changes in the media to acid and the formation of gas bubbles. For the lactose fermentation test, SR1 isolates showed negative results, while the GT2 isolates showed positive results.

Regarding the ability of isolates to hydrolyze starch or test for the presence of amylase enzymes, isolates SR1 showed positive results, while isolates of GT2 were negative. The presence of amylase enzymes allows bacteria to convert complex carbohydrates (starch) contained in organic matter into simple carbohydrates that plants or other soil organisms can use.

For the VP test, the GT2 isolate showed a positive result, while the SR1 isolate was negative. This showed that acetoin (acetyl-mehyl carbinol) compounds were detected in bacterial culture of GT2. Acetoin is an advanced product of glucose metabolism to butylene glycol. Positive results were also shown by isolates of GT2 for the simmons citrate test, while the SR1 isolate was negative. The simmons citrate test results showed that bacterial isolates GT2 were able to use citrate as a carbon source, while isolates SR1 were not. For the oxidase test, GT2 isolates also showed positive results, while SR1 isolates were negative. This showed that GT2 isolates have cytochrome oxidase enzyme activity, whereas SR1 did not. The same results were shown for the indole test. Isolate GT2 showed a positive result, while isolate SR1 was negative. This test used to determine the presence of tryptophanase enzyme activity that catalyzes the conversion of tryptophan amino acids to indole. Indole compounds themselves are precursors of the Indole acetic acid (IAA), plant growth hormone. The positive results of the indole test indicate that these bacteria have a potential characterictic to produce IAA which is important for plants.

3.6 Identification based on Bergey's manual of determinative bacteriology

Result from gram staining, endospore staining, and biochemical test were used to identify SR1 and GT2 isolates based on Bergey's Manual of Determinative Bacteriology [3]. SR1 and GT2 probably *Bacillus* sp. based on the main characteristics such as gram-positif and endospore forming bacteria, simple carbohydrate fermentation (glucose, sucrose, manitol), and facultative aerobic/anaerobic bacteria.

4. Conclusions

Of the five sampling collection sites, the highest TPC value is from the sample collected from Sokawera while the lowest is from that collected from Pageralang. The common morphological observations showed that 30 bacterial colonies were identified. The common colony configuration has a round shape, white color, and medium size. SR1 and GT2 isolates were selected based on their growth curve and generation time. Based on their tolerance, SR1 isolates may well adapt to the *buprofezin* concentration of 5 and 10 ppm, while GT2 isolate may only well adapt to that of 5 ppm.

SR1 and GT2 isolates were gram-positive and endospore forming bacteria. The biochemical tests showed the SR1 were motile, had catalase activity, can ferment glucose, sucrose, and mannitol without gas forming, unable to ferment lactose, hydrolyze starch, positive result for MR test, but negative for urease, VP test, simmons citrate, H_2S production, oxidase, and indole. The GT2 were motile, positive result for catalase test, carbohydrate fermentation (glucose, sucrose, mannitol, lactose) with gas forming, MR/VP test, simmons citrate, oxidase, and indole, but negative for starch hydrolysis, urease, and H_2S production. The SR1 and GT2 were aerobic/anaerobic facultative bacteria. SR1 and GT2 were probably *Bacillus* sp.

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