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Bioprospecting of *Bacillus pumilus* as biocontrol against fungal plant pathogens

Potensi *Bacillus pumilus* sebagai agen biokontrol jamur pathogen tanaman

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ABSTRAK

Genus *Bacillus* adalah salah satu kelompok bakteri yang dikenal sebagai *Plant Growth Promoting Rhizobacteria* (PGPR) yang dapat mendukung pertumbuhan tanaman atau mengendalikan patogen tanaman. Dalam penelitian ini dipilih tiga strain *B. pumilus* dari koleksi kultur BIOTEC (BCC 7507, TBRC 2887 dan TBRC 2903) karena berpotensi sebagai biokontrol jamur patogen tanaman. Karakteristik pertumbuhan dari strain *B. pumilus* ini ditentukan dengan membuat kurva pertumbuhan masing-masing strain. Selanjutnya, aktivitas antagonis *B. pumilus* terhadap patogen anthracnose *C. capsici, C. gloeosporioides* dan *C. acutatum*, patogen malai padi *Curvularia lunata*, patogen spot daun *Alternaria brassicicola*, dan patogen padi *Magnoporthe grisea* dievaluasi dengan mengukur zona penghambatan aktivitas mikroba. Kemudian, metabolit sekunder yang diproduksi oleh strain *Bacillus* ini juga diperiksa menggunakan uji aktivitas anti-jamur. Semua strain *B. pumilus* menunjukkan aktivitas biokontrol yang sangat rendah terhadap *M. grisea,* tetapi tidak terhadap *C. acutatum, C. Capsici, C. gloeosporioides, A. brassicicola* dan *C. lunata*. Ekstrak kultur yang berasal dari strain ini tidak menunjukkan aktivitas apa pun terhadap *M. grisea* dalam uji penghambatan perkecambahan spora.

Kata kunci: aktivitas anti-jamur, *indole asetic acid*, metabolit sekunder

ABSTRACT

The genus *Bacillus* is one of the wellknown group of bacteria as Plant Growth-Promoting Rhizobacteria (PGPR) that can support plant growth or otherwise control the plant pathogens. In this study, three strain of *B. pumilus* from BIOTEC culture collection (BCC 7507, TBRC 2887 and TBRC 2903) were investigated for their potential to be used as fungal pathogen control. The growth characteristics of these *B. pumilus* strains were determined by making the growth curve of each strain. Further, antagonistic activity of *B. pumilus* against the anthracnose pathogens *C. capsici, C. gloeosporioides* and *C. acutatum*, the rice dirty panicle pathogen *Curvularia lunata*, the leaf spot pathogen *Alternaria brassicicola*, and the rice blast pathogen *Magnoporthe grisea* were evaluated by identified the inhibition zone from their activity. Then, the secondary metabolites produced by these *Bacillus* strains were also examined using anti-fungal activity assays. All *B. pumilus* strains showed very low biocontrol activity against *M. grisea*, but not against *C. acutatum, C. Capsici, C. gloeosporioides, A. brassicicola* and *C. lunata*. Culture extracts derived from these strains did not show any activity against *M. grisea* in the spore germination inhibition assay. These results showed that the three *B. pumilus* strains did not exhibit strong potential for application as biocontrol agent.

Keywords: Antifungal, Indole Acetic Acid, secondary metabolites.

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1. Introduction

Agriculture has been developed along with the development of science. The innovation of agriculture is needed to accommodate the increase of human population. Over the past 100 years chemical fertilizers and pesticides have contributed to the improvement of crop quality and quantity (Pal and Gardener, 2006). The benefit of pesticides include improving the productivity, protection of crop losses/yield reduction, vector diseases control and increase the quality of food (Aktar 2009). However, the use of chemical fertilizers and pesticides has serious consequences, such as the risk of poisoning, development of pathogen resistance and pollution of water and soil. Long-term usage of pesticides and chemicals can lead to the ground accumulation of heavy metals such as cadmium, arsenic, and mercury beyond the limits for agricultural purposes (Yargholi and Azarneshan, 2014).

One of the most common needs for the application of chemical products in the field is for the control plant pathogens. As fungal diseases account for significant loss of agricultural crops; thus there have been considerable amount of chemical fungicide used in the field (Pal and Gardener, 2006). Anthracnose disease is a major economic constraint to chili production worldwide, especially in the tropical and subtropical regions. There are many species which caused anthracnose of chili such as *Colletotricum acutatum*, *C. gloeosporioides* and *C. capsici* (Than *et al*., 2008). *Altenaria brassicicola* is one of the most destructive fungal pathogens in Brassicaceae group in the world that caused dark spot disease (Pochon *et al*., 2012). *Magnaporthe grisea*, is a fungal plant pathogen that caused rice blast disease in rice cultivation (Howard and Valent, 1996; Talbot and Foster, 2001). Due to the widespread problem caused by the plant pathogens above, alternative solutions are needed to reduce the usage of chemical fungicides.

Member of species *Bacillus pumilus* are gram-positive, aerobic, spore-forming bacteria with diverse lifestyle. The species is closely related to *B. subtilis* and *B. licheniformis* and belongs phylogenetically to the *B. subtilis* species complex (BScs) (Rooney *et al*., 2009). Many strains of *Bacillus* and *Paenibacillus* showed an ability to suppress pests and pathogens, or otherwise support plant growth. (McSpadden Gardener and Driks, 2004). The importance of antibiotic production to plant disease suppression by *Bacillus* spp. has been demonstrated. Halophilic *B. pumilus* produces a secondary metabolite that has the ability to control fungal pathogenic species (Sewale et al. 2014). Furthermore, Zheng *et al*., (2013) reported that *Bacillus* sp. produced volatile compounds that had significant inhibitory effect on mycelia growth of *C. gloeosporioides*, including 2-nonanone, 2-decanone, gentisic acid, n-hexadecanoic acid, and 2-methylpyrazine. Further, *Bacillus* strain produce lipopeptides including pumilacidin and cyclic acylheptapeptide composed of beta-hydroxy fatty acid with antimicrobial, antiviral, and anticancer activities (Melo *et al*., 2009)

In this study, three strain of *B. pumilus* from BIOTEC culture collection (BCC 7507, TBRC 2887 and TBRC 2903) were investigated for their potential to be used as fungal pathogen control. The growth characteristics of these *B.pumilus* strains were determined.In addition, their antagonistic activity against the anthracnose pathogens *C. capsici*, *C. gloeosporioides* and *C. acutatum*, the rice dirty panicle pathogen *C. lunata*, the leaf spot pathogen *A. brassicicola*, and the rice blast pathogen *M. grisea* were evaluated. Finally, the secondary metabolites produced by these *Bacillus* strains were also examined. The results of this investigation will provide preliminary information on the potential of these *B. pumilus* strains for future biocontrol development.

2. Material and Methods

a. Preparation of Biocontrol and Plant Pathogen Strains

B. pumilus strains from BIOTEC Culture Collection were maintained in Nutrient broth (NB) and preserved as frozen stock in NB containing 20% glycerol in cryotube at -80^{o}C. For the assays, *B. pumilus* strain were streak on NA plate medium, incubated at 30^{o}C for 48 hours. One single colony were picked and dissolved into 300 μl TSB media for antagonistic assay.The target plant pathogens: *C. acutatum*, *C. capsici, C. gloeosporioides, A. brassicicola*, *M. grisea, C. lunata* were maintained on Potato Dextrose Agar (PDA) plate and kept in the dark at room temperature for further study.

b. Antagonistic Assay

The purpose of this assay was to determine the inhibitory activity of bacteria against fungal spore germination. 0.1 ml of spore suspension containing either 10^6 spores/ml for *C. acutatum* and *C. capsici*, or 10^5 spores/ml for *A. brassicicola, M. grisea* and *C. lunata* was spread on PDA plates. Bacterial samples were prepared by spotting 20 μl containing 107 CFU/ml of cell suspension onto 7 mm sterilized paper discs. Next, each disc was placed on the

center of PDA plate. The plates were incubated at 27°C for seven days. Then, the diameters of bacterial colonies and fungal growth inhibition zones were measured for the determination of fungal growth inhibition indices, according to the following equation:

$$\frac{diameter\ of\ fungal\ inhibition\ zone\ (cm)}{diameterof\ bacterial\ growth\ (cm)}$$

c. Preparation of Culture Extract

To prepare starter cultures, each of the *B. pumilus* strains was inoculated into 25 ml TSB medium and incubated at 30°C. After 6 hours incubation time, or when cell concentration reached approximately 1 x 10^6 CFU/ml, of cell suspension was transferred into 25 ml production media containing Fructose 1.6 g, Yeast extract 1.2 g, KCl 0.15 g, Distilled water 400 ml, incubated at 30°C and shaken at 200 rpm, for two days. Each culture was added with equal volume of ethyl acetate and shaken for 2 hours. Thereafter, the mixture was centrifuged at 4000 rpm for 10 minutes. The solvent phase was then separated then let dried in fume hood. The extract was subsequently dissolved with 1 ml methanol and transferred to microcentrifuge tube, then evaporated to dryness. Then, the resulting extract was weighed to determine the yield of production.

d. Anti-fungal Activity Assays

Magnaphothe grisea THL1156 (BCC 10261) was grown in Potato Dextrose Agar (PDA) and incubated at 25°C for 7 days. Then, the fungal spores were induced under black light for 3-4 days. After that, spores were harvested with 0.8% v/v Tween 20, centrifuged at 10,000 rpm for 5 minutes and re-suspended with sterilized water. Spores were then counted and diluted to 1.2 x105 spores/ml in 20 % v/v minimum salt medium (MM) (3 g/L NH4NO3, 20 g/L Glucose, 0.5 g/L KH2PO4, 0.5 g/L NaHPO4.H2O, 0.5 g/L MgSO4.7H2O, 0.5 g/L CaCl2 and 1 g/L Yeast extract).

This assay was performed in 384-well plate in triplicate. Each well was filled with 25 µl spore-suspension and left at room temperature for 2-2.5 hours to allow the spore-adhesion to wells. Subsequently, 25 µl of test compound (or positive or negative control agents) were added. Blank wells were added with 25 µl of medium and 25 µl of 10% DMSO. Plate was then incubated at 25°C for 16-18 hrs. After incubation period, plate was added with 25 µl of distilled water and 2 µl of 0.9 mg/ml CFDA in70% DMSO, and kept in dark for 5-10 minute. Plate was then washed with tap water and blotted dry on paper towels and filled with 25 µl of distilled water to each well. Fluorescence measurement was detected at 485 nm excitation and 535 nm emission wavelengths by using the bottom-reading mode of fluorometer. The signal of test wells was subtracted with that of blank wells before calculation. Percent of spore-germination inhibition was calculated by the following equation:

$$\%\ \text{Inhibition} = [1- (FUT/ FUC)] \times 100$$

Where FUT and FUC represent the mean fluorescence unit of spores treated with test compound and that treated with 0.5% DMSO, respectively. A threshold of 90% inhibition was used as a cut off for anti-fungal activity of compound, which can be classified by these criteria:

If % inhibition is < 90%, the activity is reported as “Inactive”.

If % inhibition is ≥ 90%, the activity is reported as “Active”.

3. Results

In the present experiment, the ability of three *B. pumilus* strain to control the growth of six plant pathogens were tested. The antagonistic assay was performed as described in material and method. All three *B. pumilus* strains displayed very weak activity against *M. grisea* observed as a narrow clear zone with unclear border around the paper disc containing each *Bacillus* strain (Figure 1). The average fungal growth inhibition indices of three strain *B. pumilus* against *M. grisea* from 2 experiment were almost the same, with the largest fungal growth inhibiton index was obtained from TBRC2903 (1.86), followed by TBRC2887 (1.82) and BCC7507 (1.6).On the other hand, none

of three *B. pumilus* strains showed activity toward the other fungal pathogens including *C. acutatum*, *C. capsici*, *C. gloeosporioides*, *A. brassicicola* and *C. lunata* as showed by Table 1.

Figure 1. Anti-fungal activity of *B. pumilus* strain against *M. grisea*

Table 1. Inhibition indices of *B. pumilus* strain against plant pathogens from 2 repeats of experiment.

Pathogens	*Bacillus* strain								
	BCC7507			TBRC2887			TBRC2903		
	1	2	3	1	2	3	1	2	3
C. acutatum	0	0	0	0	0	0	0	0	0
C. capsici	0	0	0	0	0	0	0	0	0
C. gloeosporioides	0	0	0	0	0	0	0	0	0
A. brassicicola	0	0	0	0	0	0	0	0	0
C. lunata	0	0	0	0	0	0	0	0	0
M. grisea	1.6	1.5	con	1.82	1.65	con	1.86	1.89	con

The crude extract of *B. pumilus* strain were produced by cultured the *B. pumilus* in production media contain 0.4% fructose, 0.3% yeast extract, and 5 mM KCl according to kyoung and sang-dal (2005) method. Based on the crude extract yield produced by all of the strain of *B. pumilus*, BCC 7507 produced the higest amount of crude extract around 7.9 mg, followed by TBRC 2903 2.2 mg and the lowest amount of crude extract produced by TBRC2887 around 0.7 mg (Table 2).

Table 2. *B. pumilus* crude extract yields

No	Strain	Crude extract yield (mg)
1	BCC7507	7.9
2	TBRC2887	0.7
3	TBRC2903	2.2
4	Control (media)	2.1

According to this result, the amount of crude extract yield did not have correlation with the activity of secondary metabolite to control plant pathogen's growth. Antagonistic assay of *B. pumilus* against *M. grisea* showed the higher activity by strain TBRC2903 and TBRC2887 compare to strain BCC7507. In contrast, the amount of crude extract yield produced by BCC7507 was higher than strain TBRC2903 and TBRC2887.

In this assay used MIC 90 determination, MIC value is defined as the lowest concentration of compound exhibiting 90% inhibition of fungal growth after exposure incubation. Based on the result of biological activity of crude extract against *M. grisea*, in contrast with antagonistic assay, it was found that all three strain of *B. pumilus* crude extract does not showed activity against the growth of *M. grisea*. It means that, the crude extact did not contain enough metabolite to inhibited *M. grisea*.

Table 3. Biological assay of crude extracts against *M. grisea* (MG 1156)

Sample	Concentration (µg/ml)	Activity against MG 1156	
		% inhibition	Result
Control	50	-97.33	Inactive
	25	-72.19	Inactive
	12.5	-53.04	Inactive
	6.25	-47.68	Inactive
	3.13	-40.30	Inactive
	1.563	-47.51	Inactive
BCC	50	-27.80	Inactive
7507	25	-84.62	Inactive
	12.5	-57.19	Inactive
	6.25	-51.29	Inactive
	3.13	-39.86	Inactive
	1.56	-44.74	Inactive
TBRC	50	-142.66	Inactive
2887	25	-76.48	Inactive
	12.5	-43.57	Inactive
	6.25	-34.16	Inactive
	3.13	-31.71	Inactive
	1.56	-34.07	Inactive
TBRC	50	-145.18	Inactive
2903	25	-100.63	Inactive
	12.5	-73.99	Inactive
	6.25	-47.11	Inactive
	3.13	-35.72	Inactive
	1.56	-33.10	Inactive

4. Discussion

From the result we can conclude that, all three strain of *B. pumilus* shows very low activity. It is possibly happened, regarding to the cells concentration used for the assay. The amount of cells needed for the antagonistic assay is not enough to inhibit fungal growth spore. Based on the method by (Czaczyk *et al*., 2000), the concentration of *bacillus* cells needed for the antagonistic assay is around 10^{10}- 10^{11} CFU/ml. Since in this experiment, the cell concentration used is aproximately about 1 x 107. Beside that, this experiment used PDA plate medium, wich is the suitable medium for fungal to grow very well. In the other side, *B. pumilus* was not be able to grow well in PDA medium, so that the bacterial cannot inhibited fungal growth. Suggested to use the medium where the bacterial as well as fungal are be able to grow in optimum condition. Zheng (2013), perfomed Antagonistic assay with 2 different media in 1 assay to test the activity of *Bacillus* strain against *C. gloeosporioides*. Bacillus strain was cultivated in NA media and *C. gloeosprioides* cultivated in PDA media. The purpose is to make the bacterial as well as fungal are be able to grow in optimum condition. The consinstency of using medium culture for bacteria during the experiment will affect to the activity of bacteria. Based on the experiment from Wang *et al*., (2009), PDA plate medium was used to culture and select *Bacillus* sp. Strain wich has activity against phatogenic fungi.

The production of metabolite is regulated by nutrients, growth rate, enzyme inactivation and induction (Demain, 1998). They are produced to help the organism in competing successfully with other organisms in their natural habitat and to adapt with the changed environmental conditions (Teasdale *et al*., 2008). In this experiment, the production medium cannot induce the *B. pumilus* strain to produce antibiotic, suggested to use another kind of production medium. This assay was perfomed to to identify the activity of bacteria against *M. grisea* using the fungal spore germination inhibition assay. Bacteria has many mechanism to control fungal pathogens growth. Based on Narayanasamy and Premchandar (2013), bacterial antagonism may be due to production of toxic metabolites (enzymes, antibiotics and volatile organic compounds), competition for

nutrients and space, prevention of pathogen colonization of host tissues and induction of resistance in plants to crop diseases. In this case, the assay may not perform the mechanisme of *B. pumilus* to inhibit the growth of fungal pathogens. Another kind of assay may be the solution to indentify the activity of *B. pumilus*.

The solvent used to extract the metabolite will affect to metabolite wich soluble in the solvent. Munimbazi and Bullermen (1998), used 40% ammonium sulphate to produced antifungal metabolites wich inhibited production of aflatoxin, CPA, OA and patulin. Further, Melo *et al*.,(2009) used hexane, dichloromethane and etyl acetate to extract antifungal from *B. pumilus*. Jung and Kim (2005), used n-butanol solvent to extract antibiotic KL39 from *B. megaterium*.The solvent used in this extraction may not extract some kind of antibiotic, wich showed by the crude extract activity assay. Based on Jung and Kim (2005) who used the same medium with this experiment to produced antibiotic said that antibiotic KL39 was insoluble in water and nonpolar organic solvent such as ethyl acetate, cloroform, n-hexane, toluena, and benzene.

5. Conclusion

All 3 strain of *Bacillus pumilus* showed very low biocontrol activity only to *M. grisea*, from antagonistic assay against all six plant pathogens (*C. acutatum, C. Capsici, C. gloeosporioides, A. brassicicola, M. grisea, C. lunata*). Secondary metabolite produced by strain *B. pumilus* did not show any activity against *M. grisea* according to inhibitory spore germination assay. In the future of study, another kind of assay should be the solution in identification of *B. pumilus* activity against plant pathogens. The production medium cannot induced the *B. pumilus* strain to produced antibiotic, suggesting to use another kind of production medium. Using another kind of solvent to extract the metabolite was also important, to find the most suitable solvent to extract a specific antifungal.

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