BIODIVERSITAS
Volume 21, Number 8, August 2020
Pages: 3431-3437

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d210803

Screening of *Burkholderia* spp. from oil palm plantation with antagonistic properties against *Ganoderma boninense*

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Manuscript received: 14 June 2020. Revision accepted: 2 July 2020.

Abstract. *Yurnaliza. Rambe DI, Sarimunggu L, Purba M, Nurwahyuni I, Lenny S, Lutfia A, Hartanto A. 2020. Screening of* Burkholderia *spp. from oil palm plantation with antagonistic properties against* Ganoderma boninense. *Biodiversitas 21: 3431-3437. Burkholderia* spp. are ubiquitous diazotrophic bacteria within β-Proteobacteria group, known for its occurrence in variety of niches from aquatic to terrestrial habitats and as endophytes. Beneficial strains of *Burkholderia* have been reported as plant growth-promoting rhizobacteria or as antagonistic bacteria against phytopathogenic fungi. This study evaluated the potential of multiple strains of *Burkholderia* spp. recovered from three ecological niches, such as rhizosphere, humus soil, and root endophytes of oil palm plantations in North Sumatra to suppress the growth of basal stem rot causative agent in oil palm (*Elaeis guineensis* Jacq.) by *Ganoderma boninense* Pat. The antagonistic isolates were identified on the basis of molecular identification using 16S rDNA sequence (27F–1462R), revealing twelve isolates (48%) as members of *Burkholderia cepacia* complex (Bcc), with other minor genera, such as *Chitinophaga, Klebsiella, Mycobacterium, Paenibacillus, Rhizobium, Serratia, Stenotrophomonas,* and *Xanthomonas*. The antagonistic activities as expressed in the percentage inhibition of radial growth (PIRG) against *G. boninense* were considerably potential with the highest percentage of 55%. In comparison, the crude extract (MeOH, EtOAc) was also tested against *G. boninense* colonies showing PIRG from 0 to 38%. Majority of isolates did not show any visible chitinolytic activity based on plate assay, in exception to *B. contaminans* RC02 while most of them were producers of glucanase. The collection of indigenous *Burkholderia* spp. originating from North Sumatran oil palm plantations, i.e. *B. cepacia, B. contaminans, B. metallica,* and *B. stagnalis* may then be considered as potential biocontrol agents against *G. boninense* based on their antagonistic activities, antifungal properties, and hydrolytic enzyme activities.

Keywords: 16S rDNA, Basal stem rot, *Burkholderia contaminans, Elaeis guineensis, Ganoderma boninense*

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a valuable tropical crop with intensive productivity in countries of Southeast region, such as Indonesia and Malaysia. Collectively, Indonesia and Malaysia produce 84% of the global palm oil with progressive trend in each year (Ommelna et al. 2012). In Indonesia, the center of oil palm plantation is located in Sumatra Island which supported the national income from the palm oil industries. Hence, the island has been proposed as a model in studying the success of oil palm growing sites in SE region with the prospect of development and manufacture of a variety of downstream products (Paterson and Lima 2017; Paterson 2019). Despite its growing popularity as a tropical oil, many challenges have been identified, from the issue of deforestation and conversion of natural sites which impact on the biodiversity, to the on-going onset of diseases such as basal stem rot (BSR) and upper stem rot (UPR) caused by the basidiomycetous fungus, *Ganoderma boninense* Pat. Regarding the diseases, it has been documented since 80 years ago and still considered as serious diseases in Indonesia (Corley and Tinker 2016).

Ganoderma boninense is categorized as the most challenging disease-causing agent which caused direct loss to the palm oil yields by infecting the bottom part of the stem leading to decay and disruption of nutrient uptake by the plant. If the diseases progress, the older fronds wilt and form a skirt around the trunk, while the plants have been considered dead under this condition (Hushiarian et al. 2013). The required step then is to promote early replanting for replacing the unproductive oil palms in the growing sites (Flood et al. 2005). Strategies in managing the disease onset have been promoted by various efforts, from early detection of infected seedlings, to direct or indirect control of the pathogenic fungus. Chemical control by injecting some fungicides to the oil palm, for example, hexaconazole and dazomet incorporated in chitosan nanoparticle were reported to effectively control the fungus growth (Maluin et al. 2019). However, the presence of dissipated and leached chemicals into the environment may pose a threat to both terrestrial and aquatic life as toxic compounds while recent development is still needed to ensure the environmental issue of the fungicides application (Maznah et al. 2015).

An alternative way to control the growth of *G. boninense* is by introducing biological control agents to diseased oil palms. This may be approached with preventive and curative methods through inoculation and application of antagonistic microorganisms capable of inhibiting the growth of *G. boninense* during certain periods of planting. A wide range of antagonistic bacteria, both from gram-positive and gram-negative genera such as *Bacillus*, *Burkholderia*, *Enterobacter*, and *Pseudomonas*, have been reported to not only inhibit the colony growth of *G. boninense* in the laboratory studies but also success in the field trials (Bivi et al. 2010; Suryanto et al. 2012; Buana et al. 2014; Irma et al. 2018). The application of bacteria as biocontrol agents is promising due to its adaptive traits in less favorable environments following the induction of systemic resistance (ISR) to the plants (Ferreira et al. 2007).

Burkholderia is a genus of diazotrophic bacteria commonly present as soil inhabitants with two different lineages either as opportunistic organismal pathogens or as beneficial microbes with plant growth-promoting traits and biocontrol potential (Compant et al. 2006). Members of *Burkholderia* have also been reported for their antifungal-producing ability to control the phytopathogenic fungus, *Ganoderma boninense*. Endophytic *Burkholderia cepacia* GanoEB2 inhibits the growth of *G. boninense* and displayed a reduction of disease incidence in 6-month oil palm seedlings planted in Malaysia (Ramli et al. 2016). Endophytic *Burkholderia* sp. B212 displayed the highest inhibition to the colony of *G. boninense* compared to other *Burkholderia* strains originating from rhizosphere region (Buana et al. 2014). Indigenous *Burkholderia* strains may be explored and studied progressively to identify the prominent isolates in suppressing the growth of *G. boninense* for future field application (Muniroh et al. 2019). To date, there is still limited information on the indigenous *Burkholderia* strains originating from the North Sumatra region. The present study reports the occurrence and diversity of *Burkholderia* spp. as the dominant bacteria group isolated from rhizosphere and humus soil in oil palm plantations with antagonistic properties against *G. boninense*.

MATERIALS AND METHODS

Study sites and collection of samples

Three oil palm plantation sites located in Medan city, North Sumatra were chosen in this study. Site I, was the oil palm plantation managed by Universitas Sumatera Utara; Site II, was the oil palm plantation managed by one of the local state-owned enterprises or PT. Perkebunan Nusantara IV (PTPN IV); and Site III, was the oil palm plantation managed by one of the local communities. Three ecological niches were designated as isolation sources for *Burkholderia* spp., such as rhizosphere, humus soil, and plant internal tissue or root endophyte. Rhizospheric soils, humus soils, and root samples of *Elaeis guineensis* were sampled adequately, stored in sterile zip-lock bags, and transported to the laboratory for further processing.

Isolation of *Burkholderia*

A nitrogen-deficient medium or Ashby's Mannitol Agar (AMA) medium for diazotrophic bacteria was used to isolate naturally-occurring *Burkholderia* strains with medium composition of (g/L): mannitol (20 g), KH_2PO_4 (0.2 g), $MgSO_4$ (0.2 g), NaCl (0.2 g), K_2SO_4 (0.1 g), $CaCO_3$ (5 g), and agar (15 g). Approximately 10 g of rhizospheric and humus soils were suspended in 90 mL distilled water and made into serial dilutions. An aliquot of 0.1 mL from each dilution was spread on top of AMA medium and incubated at 28 ^oC for 7 d. In addition, isolation of root endophytes was based on Yurnaliza et al. (2014) by surface-sterilizing the root fragments into following desinfectants such as 75% EtOH (2 min), 5.3% NaOCl (5 min), 75% EtOH (30 s), and sterile distilled water (1 min). The dried root fragments were then placed on top of AMA medium and incubated at 28 $^{\circ}C$ for 7 d. Any growing bacterial colonies were purified and preserved in fresh AMA medium until further use.

DNA amplification and 16S rDNA sequencing

The bacterial isolates were extracted for its genomic DNA following the protocol of Wizard® Genomic DNA Purification Kit (United States) based on the principle of DNA extraction by Doyle and Doyle (1987). DNA amplification in the region of 16S rDNA used a universal primer for bacterial identification (Weisburg et al. 1991), the 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers with a PCR reaction containing 8.5 µL nuclease-free water (NFW), 12.5 µL 2× GoTaq DNA Polymerase, 1 µL of each primer, 2 µL DNA template with a total volume of 25 µL. The PCR was programmed in a thermal cycler for reaction specifications such as: 94 °C (2 min), 92 $^{\circ}C$ (30 sec), 55 $^{\circ}C$ (30 sec), 72 $^{\circ}C$ (1 min), and 72 $^{\circ}C$ (5 min), with total of 40 cycles. Visible DNA amplicons with good quality from agarose gel electrophoresis were sent to Macrogen, Inc. (Singapore) for 16S rDNA sequencing.

Phylogenetic tree construction

Raw 16S rDNA consensus sequences from each isolate were subjected to BLASTn searches by selecting the optimized database for Archaea or Bacteria in the targeted loci information project (Altschul et al. 1990). The retrieved BLAST sequences were aligned using MUSCLE and constructed into a phylogenetic tree using a character-based method or maximum likelihood following the best DNA substitution model (Hasegawa-Kishino-Yano) featured in MEGA X software with a bootstrapping of 1000 replications (Hasegawa et al. 1985; Kumar et al. 2018).

Antagonistic test between *Ganoderma boninense* and *Burkholderia* isolates.

The phytopathogenic fungus, *Ganoderma boninense* was obtained from the fungal collection of Indonesian Oil Palm Research Institute (IOPRI), Medan, Indonesia, previously grown on fresh Potato Dextrose Agar (PDA) medium. The antagonistic test was based on dual culture method by measuring the percentage inhibition of radial

growth (PIRG) of *G. boninense* colony against *Burkholderia* isolates (Bivi et al. 2010). The *G. boninense* mycelial plug was placed 3 days priorly at the center of PDA medium while the *Burkholderia* isolates were inoculated on the four-edge against *G. boninense* colony using inoculation loop. The medium was incubated at 28 ˚C for 5 d. All experiments were conducted in triplicates.

Extraction of bioactive metabolites and antifungal test

Production of antifungal metabolites by *Burkholderia* isolates was performed in both liquid-state and solid-state fermentation. For liquid-state fermentation, bacterial isolates were previously grown into Nutrient Broth (NB) and incubated at 28 ˚C under 100 rpm agitation for 5 d. Solvent, EtOAc was added into the liquid medium (v/v, 1: 1) to extract the fungal metabolites by macerating it rigorously for 3 d. The top layer of solution was decanted and concentrated *in vacuo* to obtain crude extracts. For solid-state fermentation, bacterial isolates were fully streaked on Nutrient Agar (NA) medium and incubated at 28 ˚C for 5 d. The agar medium was cut into smaller fragments and immersed into 250-mL flask containing 40 mL MeOH in sufficient volume following rigorous maceration for 3d. The solution was filtered and concentrated *in vacuo* to obtain crude extracts. Both extracts were dissolved into 10% (v/v) DMSO in sterile distilled water. Similar to antagonistic test, the colony of *G. boninense* was placed 3 days priorly at the center of PDA medium while the antifungal extracts were inoculated 50 µL into sterile paper disk and placed on the four-edge against *G. boninense* colony. The medium was incubated at 28 ˚C for 5 d. All experiments were conducted in triplicates.

Detection of extracellular chitinase and glucanase using plate assays

Extracellular chitinase and glucanase produced by *Burkholderia* isolates were visually detected using plate assays. Chitinase was detected using colloidal chitin agar composed of (g/L) in sterile distilled water: 0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4.7H_20$, 0.01 g $FeSO_4.7H_2O$, 0.001 g $ZnSO_4$, 0.001 g $MnCl_2$, colloidal chitin (2 g), and bacto agar (15 g) (Yurnaliza et al. 2017). Clear zones around bacterial colonies indicate a positive result of chitinase activity. Glucanase was detected using glucan agar composed of (g/L): laminarin (10 g), agarose (8 g) in 0.1 M sodium acetate buffer (pH 5.0). Clear zones around bacterial colonies after flooding the plate with 1% congo red (5 min) and 1 M NaCl indicate a positive result of glucanase activity (Michalko et al. 2013). All detectable hydrolytic zones were measured in indexes with respect to the colony growth of each isolate.

RESULTS AND DISCUSSION

Identification of *Burkholderia* species and other genera

Twenty-five diazotrophic bacterial isolates were successfully recovered from three different niches in oil palm plantation using AMA medium. Twelve isolates (48%) were identified as species in *Burkholderia* based on 16S rDNA sequencing and BLASTn search results (data not shown). In addition, we also isolated other genera of gram-positive and negative bacteria such as *Chitinophaga* (2 isolates), *Klebsiella* (1 isolate), *Mycobacterium* (1 isolate), *Paenibacillus* (2 isolates), *Rhizobium* (3 isolates), *Serratia* (2 isolates), *Stenotrophomonas* (1 isolate), and *Xanthomonas* (1 isolate), which have also been reported to display nitrogen fixation abilities. There are different community assemblages from each niche which indicated specific preferences of bacterial species towards different environmental conditions (Figure 1). Members of *Burkholderia* were mostly isolated as soil inhabitants, both from rhizospheric and humus soil in oil palm plantations. There were no culturable *Burkholderia* species as root endophyte in our study.

The isolation medium was commonly used to isolate *Azotobacter* spp., however majority of *Burkholderia* spp. were also reported as plant-associated nitrogen fixers with diverse habitat across geographical origins (Santos et al. 2001). *Burkholderia vietnamensis* was regarded as the first N_2-fixing species among its relatives, which was isolated from the rhizosphere of rice plants (Gillis et al. 1995). Due to the increasing studies of naturally occurring *Burkholderia*, many species have been isolated as diazotrophic bacteria with prospect as plant growth promoting rhizobacteria. This study then supports the use of AMA in possibility of obtaining *Burkholderia* isolates from natural environments. The finding of none *Burkholderia* endophytic isolates may reveal that the soil environment was more favorable to the free-living lifestyle than being endophytes for the growth and enumeration of population in natural state. Specific factor in determining the community structure and diversity of soil *Burkholderia* community was the low soil acidity (pH) which was also a common feature in oil palm plantations (Von-Uexkull and Mutert 1995; Stopnisek et al. 2014).

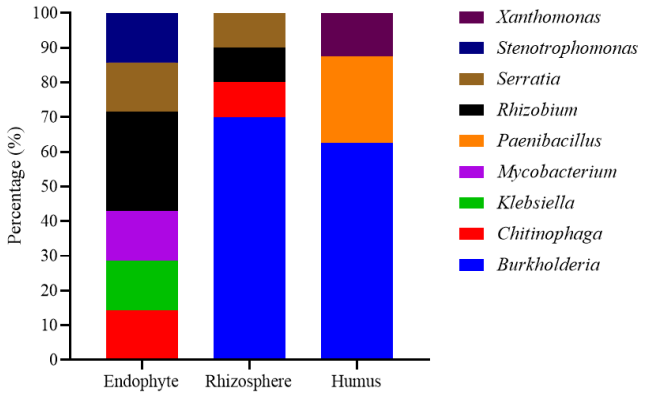

Figure 1. Community composition (%) of *Burkholderia* species and other bacteria genera isolated from three different niches in oil palm plantation

Phylogenetic relationship among *Burkholderia* isolates and GenBank database

We constructed a phylogenetic tree to illustrate the relationships among *Burkholderia* isolates based on the similarity of 16S rDNA sequences with database sequences available in GenBank. Although BLASTn resulted in high scores of identification, it may not reflect the accurate genetic relationship based on sequence alignment analysis (Koski and Golding 2001). The use of targeted loci project database was considered as valid due to the deposit of type specimens for species check and evaluation. The phylogenetic tree was then constructed using a maximum likelihood statistical method to analyze each character or nucleotides involved in evolutionary means (Figure 2). Based on the species delimitation, we identified our isolates as *Burkholderia cepacia* (1 isolate), *B. contaminans* (8 isolates), *B. metallica* (2 isolates), and *B. stagnalis* (1 isolate). All 16S rDNA sequences of each isolate have been deposited into GenBank database to obtain accession numbers (MT_565298-MT_565309).

Burkholderia contaminans is the dominant species inhabiting the soil environment of oil palm plantations in our study, followed by *B. metallica* as the second dominant and the least *B. cepacia* and *B. stagnalis*. All species belonged to the members of *Burkholderia cepacia* complex (Bcc), a group of bacteria that were considered as opportunistic pathogen but possessed beneficial biological traits for agricultural applications and biodegradation purposes (Mahenthiralingam et al. 2008). The species can be found in a wide array of environmental gradient exclusively in low pH soils. Historically, *B. stagnalis* was the newest described novel species in 2015, followed by the description of *B. contaminans* in 2009, *B. metallica* in 2008, and the oldest *B. cepacia* in 1949 (Vanlaere et al. 2008; Vanlaere et al. 2009; Smet et al. 2015). To our most understanding, the occurrence of *B. contaminans* and *B. stagnalis* in oil palm plantations may be considered as new reports for the information of other isolation sources of *Burkholderia* species.

Antagonistic and antifungal activity of *Burkholderia* isolates

Dual culture method was used to screen a large population of antagonistic *Burkholderia* isolates by inhibiting the colony growth of *G. boninense*, as expressed in PIRG (%). In general, the antagonistic test gave a higher result in PIRG (55%) to compared to the antifungal activities from metabolite extracts produced by *Burkholderia* in batch fermentation (38%) (Table 1; Figures 3 and 4). A variety of antagonistic level was observed among isolates in which the strongest antagonist was displayed by *B. contaminans* HA09 with PIRG of 55.5%, followed with *B. contaminans* HA01 (51.1%), and *B. contaminans* RC02 (47.3%). Antifungal activity of MeOH extracts showed that *B. metallica* RA01 produced the highest activity with PIRG of 38.1%, followed with *B. contaminans* HA01 (36%), and both for *B. cepacia* HB21 and *B. contaminans* RB02 (31.6%). Antifungal activity of EtOAc extracts showed that *B. metallica* RA01 produced the highest activity with PIRG of 27.2%, followed with *B. contaminans* RC03 (26.6%), and *B. contaminans* RC02 (20%). In contrast, some MeOH extracts displayed no antifungal activities compared to EtOAc although the results were higher in general.

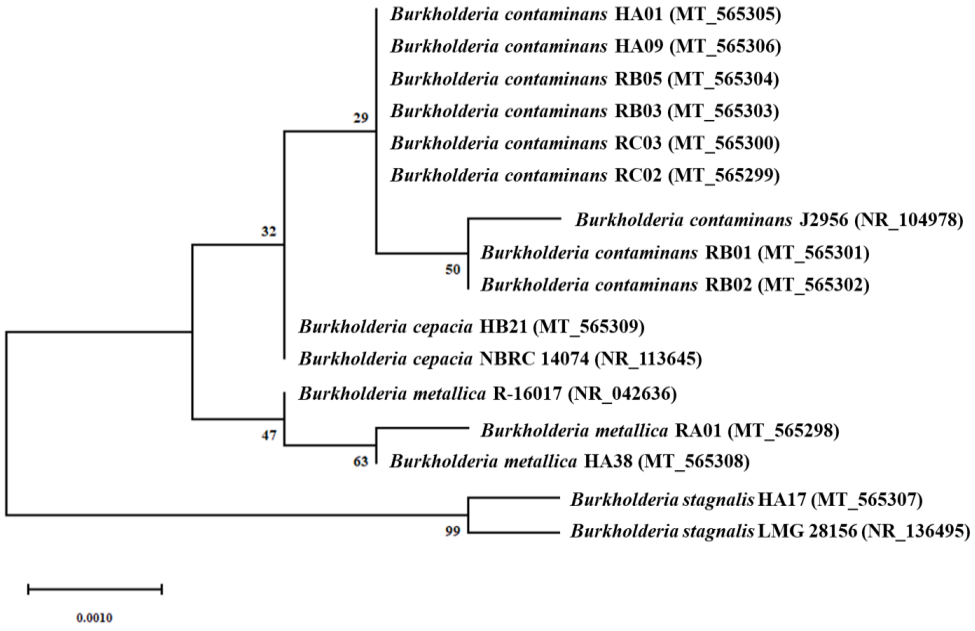

Figure 2. Phylogenetic tree of 16S rDNA sequences of the twelve isolated *Burkholderia* species using the maximum likelihood (ML) method with 1,000 bootstrap replications. The tree was constructed using MEGA X.

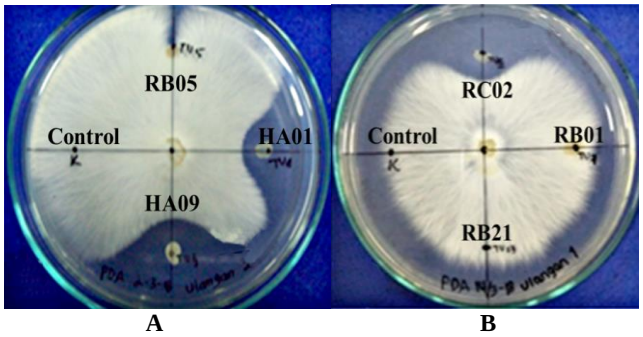

Figure 3. Antagonistic test results of representative *Burkholderia* isolates against *G. boninense* colony after incubation at 28 ºC for 5 d in PDA medium

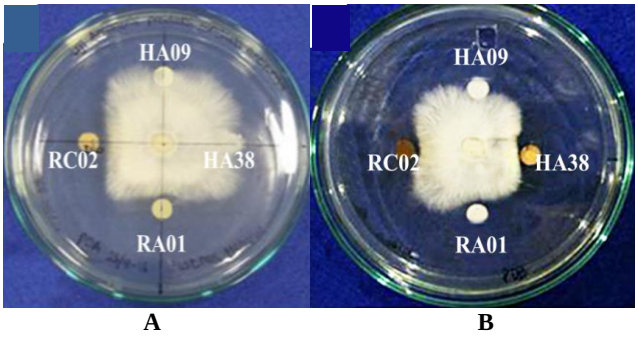

Figure 4. Antifungal test results of representative *Burkholderia* extracts against *G. boninense* colony after incubation at 28 ºC for 5 d in PDA medium. A: MeOH extracts; B: EtOAc extracts.

Table 1. Antagonistic and antifungal activities of *Burkholderia* isolates against *G. boninense* colony as expressed in the percentage inhibition of radial growth (PIRG)

Isolate	PIRG (%)		
	Anta-gonism	MeOH	EtOAc
Burkholderia cepacia HB21	31.4	31.6	6.6
Burkholderia contaminans HA01	51.1	36.0	7.1
Burkholderia contaminans HA09	55.5	-	5.3
Burkholderia contaminans RB01	-	24.0	15.8
Burkholderia contaminans RB02	31.4	31.6	6.6
Burkholderia contaminans RB03	34.2	10.5	13.3
Burkholderia contaminans RB05	-	28.8	10.5
Burkholderia contaminans RC02	47.3	21.1	20.0
Burkholderia contaminans RC03	36.8	5.2	26.6
Burkholderia metallica HA38	46.6	-	9.5
Burkholderia metallica RA01	44.4	38.1	27.2
Burkholderia stagnalis HA17	-	14.3	5.0

Table 2. Chitinolytic and glucanolytic index of *Burkholderia* isolates in plate assay

Isolate	Hydrolytic Index	
	Chitinase	Glucanase
Burkholderia cepacia HB21	-	2.5
Burkholderia contaminans HA01	-	0.5
Burkholderia contaminans HA09	-	1.7
Burkholderia contaminans RB01	-	3.1
Burkholderia contaminans RB02	-	2.6
Burkholderia contaminans RB03	-	-
Burkholderia contaminans RB05	-	2.3
Burkholderia contaminans RC02	2.7	2.6
Burkholderia contaminans RC03	-	3.1
Burkholderia metallica HA38	-	1.5
Burkholderia metallica RA01	-	2.4
Burkholderia stagnalis HA17	-	0.6

Both *Burkholderia contaminans* and *B. metallica* showed prominent and promising antagonistic activities against *G. boninense*, although *B. contaminans* displayed a higher activity based on antagonistic test collectively. The higher result in antagonistic tests may be explained by the spatial and nutrient competition in dual culture plate which later induced the *Burkholderia* strains to synthesize more versatile antifungal metabolites and hydrolytic enzymes to inhibit the growth of their competitors for the resources (Mille-Lindblom et al. 2006). Moreover, *Burkholderia contaminans* have been reported to synthesize various antifungal metabolites that were controlled by genetic regulatory elements (Gu et al. 2009). Oligopeptide produced by *B. contaminans* strain MS14 was reported to inhibit the colony growth of *Geotrichum candidum*, a human invasive pathogenic fungal species whereas the mutants with silenced gene in the region of AmbR1 produced an 80% decrease of antifungal activities when tested against the pathogenic fungus (Gu et al. 2009). In another study, it was revealed that *B. contaminans* strain MS14 also produced occidiofungin, a unique glycopeptide antifungal compound which was secreted during fermentation in liquid culture with biocontrol potential against lawn disease (Lu et al. 2009). The plant-growth-promoting strain of *B. contaminans* has also been reported through the utilization of *B. contaminans* strain KNU17BI1 isolated from maize rhizospheric soil to effectively control the onset of diseases like banded leaf and sheath blight of maize caused by *Rhizoctonia solani* (Tagele et al. 2018). The strain also displayed a broad spectrum of antagonistic activities against genera of phytopathogenic fungi such as *Alternaria, Colletotrichum, Fusarium, Pythium,* and *Stemphylium* based on laboratory test. In contrary, little is still known regarding the bioprospection study of *B. metallica* which may be further explored and exploited in the field of biological application.

Chitinolytic and glucanolytic activities from *Burkholderia* isolates

The ability to produce extracellular hydrolytic enzymes such as chitinase and glucanase may also explain the mechanisms in controlling the growth of *G. boninense* during its hyphal formation as most filamentous fungi cell walls are composed of chitin and β-1,3-glucan (Latge

2007). Chitinase acts as a hydrolytic enzyme in converting the polymeric chitin substrate into N-acetylglucosamine (NAG) monomers while glucanase acts on substrates with glycosidic type of glucans to be converted into monomeric sugars. Based on the plate assays, majority of *Burkholderia* strains were more capable of producing glucanase than chitinase as expressed in hydrolytic index (Table 2). Only *B. contaminans* RC02 was able to hydrolyze the chitin substrate as carbon source for their growth as indicated from the clear zones around colonies. Meanwhile, the highest glucanolytic index was displayed by both *B. contaminans* RB01 and *B. contaminans* RC03 with an index of 3.1, followed with *B. contaminans* RB02 (2.6), *B. contaminans* RC02 (2.6), and *B. cepacia* HB21 (2.5). Only *B. contaminans* RB03 which did not display any glucanolytic zone around its colony. The varying degree in hydrolytic activities showed that each strain was different in terms of physiological traits.

Chitinase and glucanase are key enzymes to enhance the antifungal activity of secondary metabolites produced by *Burkholderia* species. Chitinase produced by some members of *Burkholderia* was considered as unique due to its similarity with *Streptomyces* gene family, yet revealed its additional feature in disease suppression by *G. boninense* (Kong et al. 2001). Glucanase was also reported to be produced by an Indonesian strain of *B. cepacia* BiogenCC E76 isolated from rice plants with antifungal activity against *Colletotrichum gloeosporioides* (Manzila et al. 2015). Moreover, chitinase, glucanase, peroxidase (POX), and phenylalanine ammonia-lyase (PAL) are examples of pathogenesis-related proteins (PRPs) commonly involved in host defense mechanism by oil palm (Sahebi et al. 2018). Another approach is to introduce the harmless or endophytic strains of *Burkholderia* into root environment to induce systemic resistance in oil palm through increased genetic expression of PRPs to combat the *G. boninense* infection (Hushiarian et al. 2013). Previous trial has shown that the inoculation of fungal endophytes into oil palm root environment increased the chitinase activity by oil palm after 1 week of post-treatment (WPT) (Yurnaliza et al. 2017). This strategy may then be applied after significant results in field trials by using our indigenous *Burkholderia* strains in future studies. The results of our study then add the collection of indigenous *Burkholderia* species of Indonesia, especially *B. contaminans* and *B. metallica* which were still limited of their information.

ACKNOWLEDGEMENTS

This research was fully funded by the Ministry of Research, Technology and Higher Education of the Republic of Indonesia with research grant No. 48/UN5.2.3.1/PPM/KP-DRPM/2018. The authors would also express the highest gratitude to the Integrated Laboratory (*Laboratorium Terpadu*) and Laboratory of Microbiology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara for facilitating this project.

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