DETECTION OF QUORUM QUENCHING-PRODUCING BACTERIA FROM RHIZOSPHERE SOIL AS A BIOCONTROL AGENTS

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Abstract: Quorum sensing is known as a communication mechanism among bacteria to control gene expression such as bioluminescence, pigmentation, and pathogenicity. Quorum quenching is known as inhibition of quorum sensing activity. In order to block quorum sensing activity, some bacteria produced enzymes which could degrade AHL, such as AHL-acylase, AHL-lactonase, and AHL-oxidase and reductase. In this study, soil bacteria were isolated and screened for their quorum quenching activity. These isolates were divided into *Streptomyces* and non-*Streptomyces* isolates. Detection were done by using Chromobacterium violaceum as an indicator bacteria for pigment inhibition by quorum quenching activity. About 10 out of 695 isolates were shown to possess quorum quenching activity. These isolates were further identified by biochemical tests, Gram staining, and identified by 16S rRNA sequencing. Four positive isolates showed similarity with *Streptomyces* sp., and 6 positive isolates were non-*Streptomyces* which showed similarity with *Pseudomonas putida*, *Bacillus pumilus*, *Bacillus* sp., *Enterobacter ludwigii*, and *Enterobacter* sp.

Keywords: quorum quenching, quorum sensing, rhizosphere, soil, *Streptomyces*

INTRODUCTION

Pathogenic bacteria would cause infection to human host when sufficient bacteria are present to express their virulence gene. This mechanism related to quorum sensing mechanisms. In order to fight bacterial infection, researchers has found a powerful medicine, like antibiotic. Antibiotic would inhibit or kill pathogenic bacteria. However, wide use of antibiotic has inescapably resulted in the emergence of antibiotic resistance of pathogenic bacteria. Therefore, it has to be another way to fight bacterial infection. Another way to fight pathogenic bacteria is to inhibit their quorum sensing. This mechanism is known as quorum quenching. N-acyl-homoserine lactone (AHL) is an autoinducer, as signal molecule, that produced by Gram-negative bacteria. Quorum quenching compounds could inhibit quorum sensing of pathogenic bacteria so that they could not sense their cell mass and would not cause virulence to the host (Mahmoudi et

al., 2011; Kang et al., 2016; Haque et al., 2018). Quorum sensing inhibition can be done by degrading extracellular signal molecules using enzymatic activities (Abudoleh & Mahasneh, 2017).

A culturable bacteria isolated from rhiosphere soil produced AHL degradation competency (Chan et al., 2011; Achari & Ramesh, 2018). *Streptomyces* sp. were targeted as source of diverse quorum quenching agents, because these bacteria possess the capability to synthesise a variety of secondary metabolites, like hydrolytic enzymes (Tan et al., 2016; Velasco-Bucheli et al., 2020). The objective of this study was to isolate and detect rhizosphere soil bacteria from several places in Indonesia for its quorum quenching activity. This study could be used for further study of its possibility to be applied in pharmaceutical industry, especially to fight bacterial infection.

MATERIALS AND METHODS

Isolation of Rhizosphere Soil Bacteria

Rhizosphere soil samples from several places in Indonesia were collected from 15 cm depth after removing 3 cm soil surface. Soil samples were placed in plastic bags and kept at cool temperature for temporary storage. Each soil sample 1 g were placed in the oven with temperature adjusted to 70°C for an hour. Soil samples were suspended with 9 ml sterile distilled water and diluted until 10^{-2}. A total of 100 µl sample was spreaded onto Starch Casein Agar (SCA) at 25°C for 7 days and the media Luria Agar (LA) at 37°C for 1-2 days. Colonies with different morphology was isolated and purified. *Streptomyces* isolates were purified in *Streptomyces* Agar (SA), whereas non-*Streptomyces* isolates were purified in the LA media.

Detection of Quorum Quenching Activity

Quorum quenching activity was determined by well-diffusion method (Soundari et al., 2014) with modification. *Chromobacter violaceum* wild type were cultivated on Luria Broth (LB) subsequently incubated at 28°C for 24 hours. *Streptomyces* isolates were grown on *Streptomyces* Broth (SB) for 7 days at 25°C with agitation 120 rpm, whereas the non-*Streptomyces* isolates were grown in LB medium for 48 hours at 37°C. After that, the suspension was centrifuged at 7000 x g for 5 minutes. Several wells were made using sterile cork-borer. One hundred µL of *C. violaceum* (OD_{600} = 0.4) was streaked on LA using sterile cotton bud. A total of 20 µl supernatant from each isolates was inserted into each wells and the plates were incubated at 28°C for 24 hours. SB and LB were used as negative control. Quorum quenching activity was detected by inhibition of violacein production (no purple-pigmented colonies) around the wells.

Biochemical Test and Gram Staining

Biochemical test for *Streptomyces* isolates are motility, indole test, and catalase. Biochemical test for non-*Streptomyces* isolates are motility, indole test, catalase and oxidase test, urease production, citrate utilisation, hydrogen sulfide test (TSIA), and lysine decarboxilase. Gram staining were done to differentiate bacteria into Gram classification, and also to determine their morphology.

Molecular Identification of Rhizosphere Soil Bacteria

Total DNA genome extraction of positive isolates for quorum quenching activity were done using Promega DNA Purification Kit and used as DNA template for PCR

amplification of 16S rRNA gene. The identification of the isolates was conducted using universal primer, namely 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387R (5′-GGGCGGWGTGTACAA GGC-3′) (Marchesi et al., 1998). The 50 µL reaction mixture contained of 0.5 µL DNA template (100 ng), 2 µL of forward primer 63F, 2 µL reverse primer 1387R, 25 µL of Go Taq® Green (Promega) 2x, and 20.5 µL of ddH_2O.

PCR condition started with pre-denaturation at 94°C for 5 minutes. All cyclers were designed to perform 30 cycles consisting of denaturation at 94°C for 1 minutes, annealing at 56°C for 1 minute, and extension at 72°C for 1.5 minutes followed by a final extension step of post-extension at 72°C for 5 minutes. PCR products were visualized by agarose gel electrophoresis in 1% agarose gel at 90 Volt for 60 minutes using 1x TAE buffer, visualized under UV light with the help of FloroSafe DNA Stain (1^{st} BASE), and recorded with Gel Doc instrument (BioRad, USA).

PCR results of 16S rRNA gene was purified and sequenced to determine the nucleotide order of DNA fragments of each isolate. Purification and sequencing were performed by Macrogen, Korea using ABI3730XL. Data analysis were done using BLAST program from National Centre for Biotechnology Information (NCBI) to identify positive isolates.

RESULTS AND DISCUSSIONS

Isolation and Detection of Quorum Quenching Activity

A total of 60 rhizosphere soil samples from several regions in Indonesia were screened for *Streptomyces* and non-*Streptomyces* isolates. In this study, 362 *Streptomyces* isolates and 333 non *Streptomyces* isolates were successfully isolated. These isolates were screened for its quorum quenching activity towards *C. violaceum* wild type as indicator bacteria.

Results showed that only 1.44% of total rhizosphere soil bacterial isolates were positive for quorum quenching activity towards the indicator bacteria (Figure 1). These isolates were isolate D, (1), SH21, PKS03, H, LSY13, FLB12, PB01, MKJ12, and PK01. Isolate D, (1), SH21, and PKS03 were characterized as *Streptomyces* from their morphology and growth in the cultivation medium, as the other isolates were characterized as non-*Streptomyces*. This results is consistence with previous research about the presence of quorum quenching activity from soil bacteria (Chan et al., 2011; Mahmoudi, Tabatabaei, & Venturi, 2011; Chong et al., 2012; Chankhamhaengdecha et al., 2013; Hassan et al., 2016) This result was similar to previous study that most positive isolates were originated from turf soil or agricultural soil in plateau area (Wang & Leadbetter, 2005).

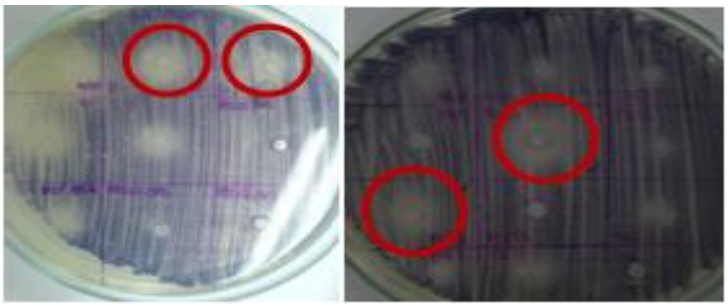

Figure 1. Quorum quenching activity of *Streptomyces* isolates (shown with red circle)

Biochemical Test and Gram Staining

Positives isolates for quorum quenching were further identified by biochemical test and Gram staining. Characteristics of *Streptomyces* isolates were positive for hydrolysis casein, indole negative, and catalase positive (Table 1). Non-Streptomyces isolates showed different biochemical characteristics and morphology. Most of non-*Streptomyces* isolates were rod-shaped Gram negative bacteria (Table 2).

Table 1. Biochemical test of *Streptomyces* isolates from rhizosphere soil

Isolates code	Casein hydrolysis	Indole	Catalase
D	(+)	(-)	(+)
(1)	(+)	(-)	(+)
SH21	(+)	(-)	(+)
PKS03	(+)	(-)	(+)

Table 2. Biochemical test of non-*Streptomyces* isolates from Rhizosphere soil

Isolates Code	Catalase	Oxidase	Citrate	Urease	TSIA	LD	Motility	Indole	Gram Staining
H	++	-	+	-	A/A ≠ gas	+	Motile	-	Gram (-) Bacilli
LSY13	-	+	-	-	A/K ≠ gas	+	Motile	-	Gram (-) Bacilli
FLB12	-	-	+	+	K/A ≠ gas	+	Motile	-	Gram (-) Bacilli
PB01	+++	-	+	-	K/A ≠ gas	-	Non motile	-	Gram (+) Bacilli
MKJ12	-	+	+	-	K/K ≠ gas	-	Motile	-	Gram (-) Bacilli
PK01	+	+	+	+	A/A ≠ gas	-	Non motile	-	Gram (+) Bacilli

A: Acid, K: Alkaline, LD: Lysine Decarboxilase

Molecular Identification of Rhizosphere Soil Bacteria

PCR amplification of the 16S rDNA gene generated the amplicon size about 1300 bp (Figure 2). Isolate D, (1), SH21, and PKS03 similar to the genus *Streptomyces* sp (Table 3). In previous study, *Streptomyces* sp. produced extracellular AHL-acylase encoded by *ahl*M gene and could inhibit the violacein production of indicator bacteria (Sakr et al., 2015; Velasco-Bucheli et al., 2020).

GenBank database suggested isolate FLB12 and H belong to the genus *Enterobacter* sp. Isolate FLB12 share 97% sequence identity with 16S rRNA gene of *Enterobacter ludwigii* strain BIHB 336, whereas isolate H share 99% sequence identity with 16S rRNA gene of uncultured *Enterobacter* sp. clone F4aug.22. According to the database, isolate PB01 share 96% similarity to *Bacillus pumilus* strain JMC11, whereas isolate PK01 share 75% similarity to *Bacillus* sp. G2-10 (Table 3). *Bacillus* sp. was known for their quorum quenching activity by producing AHL-lactonase which could inhibit violacein production of *C. violaceum* (Yin et al., 2012). This enzyme successfully interrupt the quorum sensing system of *Aeromonas hydrophila* (Chu et al., 2014; Chen et al., 2019; Zhou, Yu, & Chu, 2019), *Pseudomonas aeruginosa* (Raafat, Ali-Tammam, & Ali, 2019), and *Vibrio parahaemolyticus* DAHP1 (Vinoj et al., 2014).

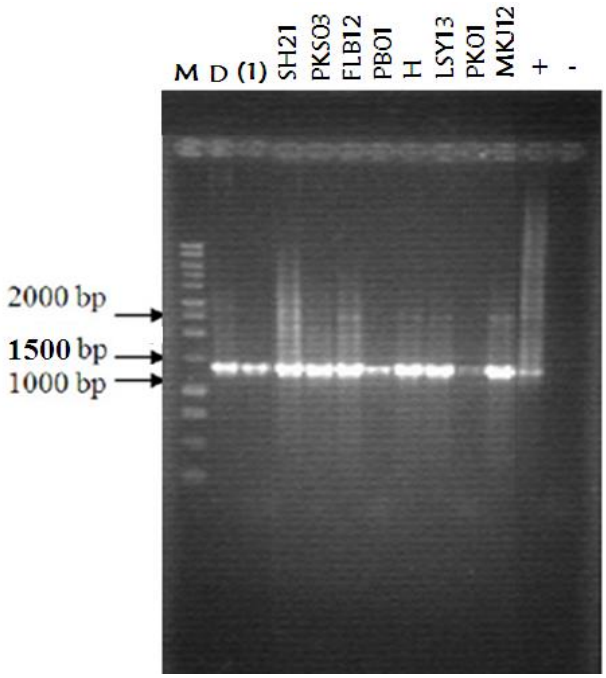

Figure 2. PCR amplification of positive isolates 16S rRNA genes Lane M: 1 kb DNA ladder. Positive control: bacterial isolates positive for 16S rDNA gene; negative control: PCR mixtures without DNA template. The targeted amplicon size is 1300 bp.

Isolate LSY13 and MKJ12 share 99% sequence identity with 16S rRNA gene of *Pseudomonas putida* strain TP0701 (Table 3). *Pseudomonas* sp. 1A1 was able to produce AHL-acylase and disrupted a broad range of AHLs (Cheong et al., 2013).

Table 3. Identification of positive isolates for quorum quenching activity

Isolate	Rhizosphere soil sample	Location	Soil description	Similarity	Max Ident
D	Mango apple tree	Cileungsi	Turf soil	*Streptomyces* sp. 2438	87%
(1)	Bananas tree	Cileungsi	Turf soil	*Streptomyces* sp. 2438	95%
SH21	Zallaca palm	Situgunung	Forest soil	*Streptomyces* sp. 2438	84%
PKS03	*Livistonia* sp.	Situgunung	Turf soil	*Streptomyces* sp. BC37	88%
FLB12	Poinciana tree	Borobudur	Turf soil	*Enterobacter ludwigii* strain BIHB 336	97%
PB01	Palm tree	Bogor	Turf soil	*Bacillus pumilus* strain JCM11	96%
H	Pine tree	Cileungsi	Turf soil	Uncultured *Enterobacter* sp. clone F4aug.22	99%
LSY13	*Leucaena leucocephala*	Sleman	Agricultural soil	*Pseudomonas putida* strain TP0701	99%
PK01	Papaya tree	Kaliurang	Agricultural soil	*Bacillus* sp. G2-10	75%
MKJ12	Mango tree	Jakarta	Turf soil	*Pseudomonas putida* strain TP0701	99%

Quorum quenching activity of rhizosphere soil bacteria could be used for further study for its application. Quorum quenching mechanism are proposed as an alternative to attenuate and fight bacterial infection, and also direct application of AHL-degrading bacteria as biocontrol agents against phytopathogenic bacteria (Garge & Nerurkar, 2017;

Ha et al., 2018; Alinejad et al., 2020; Vesuna & Nerurkar, 2020).

CONCLUSIONS

In this study, ten soil bacterial isolates were known to possess quorum quenching activity towards indicator bacteria *C. violaceum*. Most of these bacteria were originated from rhizosphere soil samples which were taken from plateau. The ability of soil bacteria to perform quorum quenching mechanism as shown in this study could be used for further study of its possibility to be applied in pharmaceutical and agricultural industries.

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