



**AKTIVITAS IN VITRO ANTIBIOFILM DARI BAKTERI ASAL AIR
TERJUN DAN LAUT TERHADAP BAKTERI PATOGEN MANUSIA**
**THE IN VITRO ANTIBIOFILM ACTIVITY OF BACTERIA ISOLATED FROM WATERFALL AND
MARINE ENVIRONMENT AGAINST HUMAN BACTERIAL PATHOGENS**

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Abstrak

Keterlibatan biofilm pada infeksi kronis dan pada permukaan peralatan medis selalu menjadi wacana penting bagi kesehatan umum di dunia. Biofilm bakteri berkaitan dengan tingkat resistensi terhadap antibiotik yang menjadikan infeksi sulit untuk diobati. Untuk mengatasi masalah ini, pengendalian yang efektif perlu diimplementasikan, seperti penerapan senyawa antibiofilm. Beberapa tahun terakhir, lingkungan akuatik menjadi salah satu sumber potensi penghasil senyawa bioaktif, termasuk senyawa antibiofilm. Tujuan dari penelitian ini yaitu menapis dan mengkarakterisasi bakteri asal air terjun dan laut yang diperoleh dari beberapa lokasi di Indonesia, sebagai penghasil aktivitas antibiofilm. Isolat dievaluasi berdasarkan kemampuan aktivitas antimikroba terhadap enam bakteri patogen dan diikuti dengan penapisan senyawa antibiofilm. Sebanyak 11 dari 65 isolat menunjukkan aktivitas *quorum sensing* atau *quorum quenching*, dan hanya terdapat satu isolat yang memiliki aktivitas keduanya. Supernatan kesebelas isolat menunjukkan penghambatan pembentukan biofilm setidaknya terhadap satu patogen dengan metode uji biofilm statis. Karakterisasi senyawa bioaktif dari lima isolat yang terpilih menunjukkan aktivitas senyawa yang berbeda, seperti karbohidrat, protein, dan asam nukleat. Sekuensing gen penyandi 16S rRNA menetapkan kelima isolat tersebut berada dalam dua genus yang berbeda, *Vibrio* (WK2.4, WK2.6, and WK2.3) dan *Pseudomonas* (S1.2 dan S1.3). Penelitian ini memberikan wawasan baru terhadap pencarian kandidat bakteri akuatik sebagai agen antibiofilm yang potensial.

Kata kunci: Air terjun; Antibiofilm; Bakteri laut; *Quorum quenching*

Abstract

Biofilm involvement in chronic infections and on the surface of medical equipment have been considered as public health concern worldwide. Bacterial biofilm is related to antibiotic resistance, making the diseases difficult to treat. An effective control strategy should be implemented, for example, by applying antibiofilm agents. The use of aquatic environment as potential sources of bioactive compounds, including the antibiofilm compounds, is recently of concern. This study aimed to screen and characterize bacteria with antibiofilm activity that were isolated from waterfall and marine environment and obtained from several locations in Indonesia. The isolates were firstly evaluated for their antimicrobial activity against six bacterial pathogens and followed by antibiofilm screening. Eleven out of 65 isolates showed quorum sensing or quorum quenching activity, and one of them showed both activities. Supernatants of 11 isolates inhibited biofilm formation of at least one pathogen by using static biofilm assay. Bioactive compounds characterization of the selected five isolates revealed the presence of different compounds, such as carbohydrates, proteins, and nucleic acids. The 16S rRNA gene sequencing analysis classified five isolates into two different genera, namely *Vibrio* (WK2.4, WK2.6, and WK2.3) and *Pseudomonas* (S1.2 and S1.3). The present study provides insights into the discovery of aquatic bacteria candidates as antibiofilm agents.

Keywords: Antibiofilm; Marine bacteria; *Quorum quenching*; Waterfall

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INTRODUCTION

Biofilm is a consortium of microorganisms embedded in a self-produced matrix of extracellular polymeric substances on surfaces. Biofilm formation allows cells to adapt and survive in hostile environments. With regard to the medical aspect, biofilm poses serious public health issues as it is able to colonize human body and medical devices. Bacterial biofilm has high resistance to antibiotics and host immune systems, hence biofilm-associated infections are difficult to treat (Vu, Chen, Crawford, & Ivanova, 2009) and eradicate completely (Lewis, 2001). Some examples of these infections are catheter-associated urinary tract infections, endocarditis, and chronic rhinosinusitis (Lebeaux, Chauhan, Rendueles, & Beloin, 2013). Besides, the following pathogens are frequently involved in biofilm infections, *Acinetobacter baumannii* (Longo, Vuotto, & Donelli, 2014), *Burkholderia cepacia* (Coenye, 2010), *Escherichia coli* (Trautner & Darouiche, 2004), *Pseudomonas aeruginosa* (Rasamiravaka, Labtani, Duez, & El Jaziri, 2015), *Salmonella enterica* (Koopman et al., 2015), and *Staphylococcus aureus* (Yarwood, Bartels, Volper, & Greenberg, 2004).

Antibiofilm agents with their unique mechanisms to thwart biofilm formation could be regarded as a potential strategy to tackle the biofilm problems. The inhibition of cell attachment to surfaces is the main strategy to prevent further development of biofilm (Rabin et al., 2015). Another approach focuses on bacterial intercellular communication or quorum sensing system since its mechanism plays an important role in biofilm development. This signal interference, also known as quorum quenching, would affect the expression of virulence factors required for bacteria to switch to sessile states (Brackman & Coenye, 2015).

Aquatic environment, especially marine, serves as a reservoir for a vast diversity of bacteria, making it a valuable resources of bioactive compounds, such as antimicrobial (Anand et al., 2006), antifungal (Woo, Kitamura, Myouga, & Kamei, 2002), antibiofouling (Waturangi, Hariyanto, Lois, Hutagalung, & Hwang, 2017), and antibiofilm agents (Waturangi, Bunardi, & Magdalena,

2011; Papa et al., 2015; Camesi, Lukito, Waturangi, & Hwang, 2016). Waterfall environment also seems to be a promising source of biologically active compounds. A recent study has revealed the quorum quenching activity of a bacterium, *Labrenzia* sp. BM1, isolated from Malaysian waterfall (Ghani, Norizan, Chan, Yin, & Chan, 2014). Despite this finding, waterfall environment is considered as an untapped source by reason of limited research in this area. To the best of our knowledge, this is the first study to explore the antibiofilm activity of bacteria from waterfall. This study aimed to screen and characterize bacteria with antibiofilm activity against bacterial pathogens commonly involved in biofilm-associated infections that were isolated from waterfall and marine environment and obtained from several locations in Indonesia.

MATERIAL AND METHODS

Sampling and Isolation of Bacteria

Water samples were collected from several waterfalls and seas in several provinces in Indonesia (Bangka-Belitung Islands, Bali, Banten, West Java, DKI Jakarta and North Sulawesi) (Table 1). Samples from waterfall were taken directly from the ledge part of waterfall, while samples from marine environment were taken at around 300 meters away from the shoreline. The samples were labelled and transported to the laboratory. Each sample was then serially diluted and spread on the respective media, Nutrient Agar (NA) (Oxoid, Basingstoke, UK) for waterfall samples and Marine Agar (MA) (Difco, Detroit, USA) for marine samples. All media were incubated at 28 °C overnight and bacterial colonies with observable different morphology and color were isolated.

Bacterial Strains and Culture Media

For screening of quorum sensing and quorum quenching activity, *Chromobacterium violaceum* CV026 and *C. violaceum* Wild Type from Atma Jaya Culture Collection were used as reporter strain. The former was a mutant which could not produce N-hexanoyl-L-homoserine lactone (C6-HSL), yet was able to induce violacein production in response to autoinducer molecules in its environment (McClellan et al., 1997). Both bacteria were

obtained from Atma Jaya Culture Collection and routinely cultured on Brain Heart Infusion Agar (BHIA) (Oxoid, Basingstoke, UK) at 28 °C. Six bacterial pathogens were also selected as tested bacteria: *Acinetobacter baumannii* ATCC 19606, *Burkholderia cepacia* ATCC 25416, *Escherichia coli* ATCC 4157, *Pseudomonas aeruginosa* KCTC 1637, *Salmonella enterica* ATCC 51741, and *Staphylococcus aureus* ATCC 25923. All tested bacteria were cultivated on Luria Agar (LA) (tryptone 10 g, sodium chloride 5 g, yeast extract 5 g, agar 20 g, and ddH₂O 1,000 mL) at 37 °C, except for *B. cepacia* which was grown at 30 °C.

Crude Extract Production

Each isolate from waterfall and marine environment was cultured in nutrient broth (NB) (Oxoid, Basingstoke, UK) and sea water complete (SWC) (peptone 5 g, yeast extract 1 g, glycerol 85% 3 mL, sodium chloride solution 3% 750 mL, and ddH₂O 250 mL), respectively, at 28 °C for 48 hours. The suspensions were adjusted to achieve absorbance value of 0.132 at $\lambda = 600$ nm (McFarland 0.5), then centrifuged twice at 10,016 x g (Thermo Scientific) for 10 minutes to remove residual bacterial cells. The supernatant was collected and filtered using 0.22 μ m pore size filter to remove residual bacterial cells. These crude extracts were stored at 4 °C for a week and at -20 °C for a month.

Antimicrobial Assay

The assay was performed in duplicate using agar well diffusion method. Each tested bacteria was cultured in Luria broth (LB) at their respective optimum temperature until reaching absorbance value of 0.132 at $\lambda = 600$ nm. Afterward, 100 μ L of tested bacteria was spotted and swabbed (with cotton swab) three times on Mueller-Hinton Agar (MHA) (Oxoid, Basingstoke, UK). Plates were divided into nine sections and wells were made using sterile cork borer. As much as 20 μ L of previous supernatant was loaded into the wells. Antibiotic disk of nalidixic acid (30 μ g/mL) and trimethoprim-sulfamethoxazole (25 μ g/mL) were used as positive controls, whereas uninoculated media were used as

negative control. All cultures were incubated according to the growth temperature of tested bacteria and examined after 24 h. Clear zones of growth inhibition were interpreted as positive results.

Screening of Quorum Sensing Activity

According to Boşgelmez-Tinaz, Ulusoy, Aridoğan, Eroğlu, and Kaya (2005), bacterial isolates and *C. violaceum* CV026 were grown in parallel streak on BHIA (Oxoid, Basingstoke, UK). *C. violaceum* was used as positive control in this screening. Cultures were then incubated at 28 °C. The appearance of purple pigmentation after 48 hours indicated quorum sensing activity of isolates. Experiments were conducted in triplicates.

Screening of Quorum Quenching Activity

Quorum quenching activity was detected using agar well diffusion method which is a modification of the procedure of Chong et al. (2012). *C. violaceum* was grown in Brain Heart Infusion Broth (BHIB) (Oxoid, Basingstoke, UK) at 28 °C with shaking at 120 rpm until reaching absorbance value of 0.5 at $\lambda = 600$ nm (approximately 10⁸ CFU/mL). Subsequently, 100 μ L of *C. violaceum* was spotted and swabbed three times on BHIA (Oxoid, Basingstoke, UK). Plates were divided into nine sections and wells were made using sterile cork borer. 20 μ L of each supernatant was loaded into the wells and uninoculated media were used as negative control. The plates were incubated at 28 °C for 48 hours. Quorum quenching activity was shown by reduction or disappearance of purple pigments, but viable cells around the wells (Vasavi, Arun, & Rekha, 2015). Measurements were made from the outer edge of the wells to the edge of the clear zones. Experiments were conducted in triplicates.

Biofilm Inhibition Activity Assay

The assay was done using static biofilm assay as described by Camesi et al. (2016) with modifications. The tested bacteria were grown in BHIB (Oxoid, Basingstoke, UK) at their respective incubation temperature until reaching absorbance value of 0.132 at $\lambda = 600$ nm (McFarland 0.5). Approximately 200 μ L of tested bacteria was placed in 96-wells

microplate along with 5% (v/v) crude extract. About 200 μ L of tested bacteria was separately used as positive control, while 200 μ L of uninoculated media was used as negative control. After an overnight incubation, media and planktonic cells were discarded. The wells were rinsed twice with sterile distilled water and allowed to dry before proceeding into staining. Around 200 μ L of crystal violet 0.4% was used to stain each well which was further being incubated for 30 minutes. The dye was discarded, then each well was rinsed three times using sterile distilled water and air dried. The stained biofilm was solubilized with 200 μ L of absolute ethanol for 30 minutes. Finally, the solubilized crystal violet was transferred to a new microplate and the optical density was determined at 595 nm using the model 680 microplate reader (Bio-Rad, California, USA). The percentage of biofilm inhibition was calculated using the following equation: $[(\text{OD growth control} - \text{OD sample})/\text{OD growth control}] \times 100$ (Sandasi, Leonard, & Viljoen, 2010).

Microscopic Observation and Biochemical Tests

Isolates were stained using Gram staining and observed under magnification of 100 x 10 for microscopic observation. The biochemistry tests used in this study included carbohydrate fermentation broth (glucose, lactose, maltose, and mannitol), Triple Sugar Iron Agar (TSIA), citrate, and catalase. Additionally, marine bacteria were tested for their ability to grow on different NaCl concentrations (0, 1, 3, and 6%).

Bacterial Strain Identification

Bacterial genomic DNA from bacterial cultures was extracted using Wizard® Genomic DNA Purification Kit (Promega, Wisconsin, USA) according to the manufacturer's instructions and the resulting DNA was used as template for PCR. The identification of the strains was carried out by amplifying the 16S rRNA gene using primer sequences 63F (5'-CAGGCCTAACACATGC AAGTC-3') and 1387R (5'-GGGCGGWTGT ACAA GGC-3') (Marchesi et al., 1998). The components of PCR reaction mixture were 4 μ L of DNA template, 2 μ L of each primer, 25 μ L of Go Taq® Green (Promega, Wisconsin,

USA) 2x, and 17 μ L of nuclease-free water. The PCR protocol was: pre-denaturation at 94 °C for 5 minutes, denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute, followed by post-extension at 72 °C for 20 minutes; 30 cycles. PCR products were further analyzed by gel electrophoresis in 1% agarose gel and 1 kb DNA ladder was used as the marker. The electrophoresis was carried out at 90 volt for 60 minutes using TAE 1x buffer. The gel was visualized under UV light and recorded with Gel Doc instrument (Bio-Rad, California, USA). PCR results were sent to 1st BASE, Malaysia for sequencing. The sequences were compared with GenBank databases using the BLAST program (NCBI) and submitted to GenBank to obtain accession numbers.

Characterization of Bioactive Compounds

The characterization was conducted following the method of Jiang et al. (2011). Crude extracts of each isolate were treated with NaIO₄ (Sigma-Aldrich, Missouri, USA) 20 mM, proteinase-K (Biolone, London, UK) 1 mg/mL, and nuclease [DNase (Sigma-Aldrich, Missouri, USA) 100 μ g/mL and RNase (Sigma-Aldrich, Missouri, USA) 25 μ g/mL] separately and incubated at 37 °C for 12 hours. The treated crude extracts were used in static biofilm assay.

RESULTS

Isolation and Antimicrobial Activity of Bacteria from Waterfall and Marine Environment

Sixty five isolates in total were obtained from the selected samples, 18 strains from waterfall and 47 strains from marine environments (Table 1). Agar well diffusion method was used to evaluate the antimicrobial activity of all isolates. This step aimed to ensure that antibiofilm activity of isolates was not resulted from growth inhibition. None of the tested isolates showed growth inhibition zones against the tested bacteria.

Screening of Quorum Sensing and Quorum Quenching Bacteria

All isolates were screened for quorum sensing (Figure 1a) and quorum quenching activities (Figure 1b) using reporter strain *C.*

violaceum CV026 and *C. violaceum*, respectively. Purple pigmentation is regulated by quorum sensing activity. *C. violaceum* CV026 is a mutant that cannot produce N-hexanoyl-L-homoserine lactone (C6-HSL). This signal molecule is essential in expression of gene that responsible for purple pigmentation. The presence of other bacteria-producing N-hexanoyl-L-homoserine lactone, will turn the colour of mutant colony into purple. Among these, quorum sensing activity was shown by seven isolates (AN1.1, B1.1, WM1.3, WK2.1, WK2.3, WK2.4, and WK2.6).

On the contrary, five isolates (S1.1, S1.2, S1.3, S1.4, and WK2.3) showed quorum quenching activity and the diameter of clear zones were 2 to 3 mm. *C. violaceum* within the zones was streaked as before to another plate to ensure that clear zones were not resulted from antibacterial activity (Figure 2). Isolate

WK2.3 particularly displayed both quorum sensing and quorum quenching activities.

Inhibition of Initial Biofilm Development

Eleven isolates which showed positive results in the screening of quorum sensing, quorum quenching, or both abilities were tested to confirm whether they could inhibit biofilm formation by several tested bacteria using static biofilm assay. As a result, all isolates were found to have antibiofilm activity against at least one tested bacteria. Crude extract of S1.3 had the highest antibiofilm activity against *B. cepacia* and *P. aeruginosa*, while extract of WK2.1 had biofilm activity against *A. baumannii* and *S. enterica*. Moreover, WK2.4 and WK2.6 had the highest activity to inhibit biofilm formation of *S. aureus* and *E. coli*, respectively (Table 2).

Table 1. Bacterial isolates from waterfall and marine environment in Indonesia

Origin	Sample code	Number of isolates
Cikahuripan Waterfall, West Java	AN	2
Bibijilan Waterfall, West Java	CB	6
Mata Air Darmaloka, West Java	S	10
Pulau Pramuka Beach, DKI Jakarta	N	10
Perawan Beach, DKI Jakarta	V	14
Putri Beach, DKI Jakarta	LP	4
Tanjung Pasir Beach, Banten	A	2
Padang Padang Beach, Bali	SS	2
Bunaken Beach, North Sulawesi	BU	4
Matras Beach, Bangka Belitung Islands	WM	4
Ketawai Beach, Bangka Belitung Islands	WK	5
Rambak Beach, Bangka Belitung Islands	WR	2
Total number		65

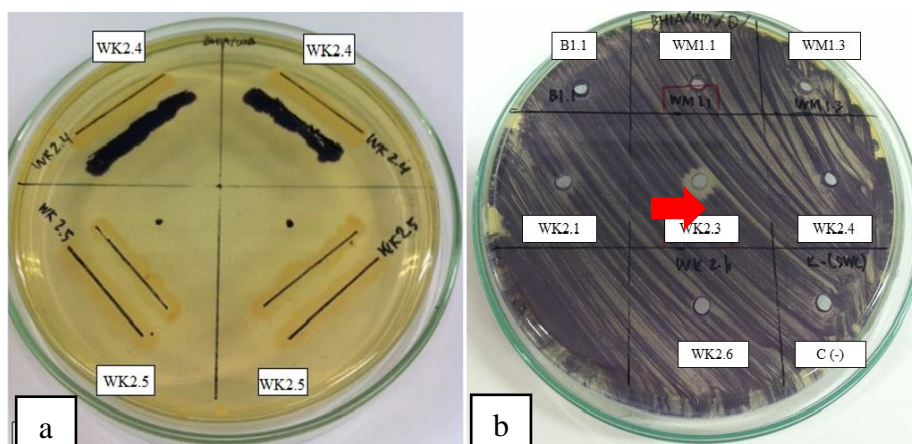


Figure 1. Quorum sensing and quorum quenching activities of isolates (shown with arrows). (a) WK2.4 showed quorum sensing activity by inducing violacein production, (b) WK2.3 showed quorum quenching by forming clear zone

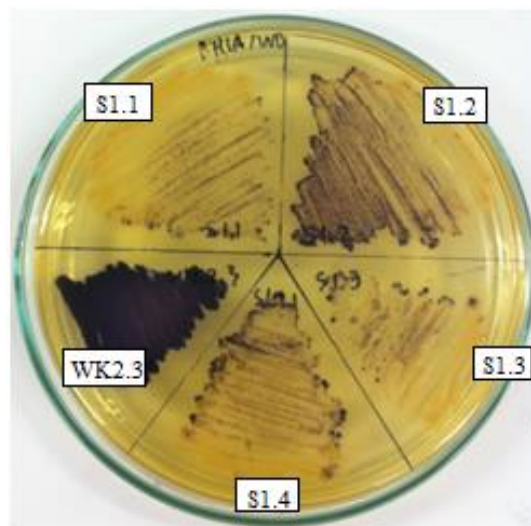


Figure 2. Viable *C. violaceum* bacterium after being re-streaked back from clear zones area

Table 2. Biofilm inhibition activity (%) using 5% (v/v) crude extracts

Isolate	Inhibition (%)					
	<i>A. baumannii</i>	<i>B. cepacia</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. enterica</i>	<i>S. aureus</i>
WM1.3	40.99	6.39	-	0.16	-	44.91
WK2.1	72.69	17.18	-	7.95	44.89	46.27
WK2.4	8.68	17.87	48.88	2.57	42.98	80.30
WK2.6	53.44	37.44	95.97	13.58	24.53	-
AN1.1	-	4.10	31.69	-	-	-
B1.1	-	39.25	-	-	-	-
S1.1	-	-	-	34.43	-	74.21
S1.2	28.51	-	-	13.12	16.90	14.89
S1.3	27.55	42.77	13.56	39.69	75.40	71.08
S1.4	45.79	-	61.71	-	-	-
WK2.3	12.79	28.81	48.68	8.32	42.89	19.44

Five out of 11 isolates (WK2.4, WK2.6, S1.2, S1.3, and WK2.3) were then selected for further study. These isolates were chosen as representative of three types of screening results and also on the basis of the largest number of tested bacteria which showed antibiofilm activity. Although WK2.1 and WK2.6 exhibited antibiofilm activity against five tested bacteria, the latter was preferred due to its high activity toward *E. coli*.

Characterization of Bioactive Compounds

Pre-treatment of each crude extract with NaIO₄, proteinase-K, and nuclease could reduce its antibiofilm activity against tested bacteria, thus enabled us to identify the bioactive compounds which involved in such activity. As depicted in Figure 3a, pre-treatment extract of WK2.6 with proteinase-K and nuclease resulted in a decreasing antibiofilm activity against *A. baumannii*.

Figure 3b shows that the activity of extract against *B. cepacia* decreased after being treated with nuclease. However, none of the pre-treatments could decrease the extract's activity against *S. enterica* (Figure 3c).

Following these results, it is inferred that bioactive compounds accounted for antibiofilm activity of extract WK2.6 are protein and nucleic acid for *A. baumannii*, nucleic acid for *B. cepacia*, and none for *S. enterica*. In summary, different compounds were involved in antibiofilm activity of each isolate against particular tested bacteria (Table 3).

Identification of Isolates

All five isolates were stained and observed using microscope. Biochemical tests were then applied to determine metabolic properties of the isolates. Finally, 16S rRNA sequencing was carried out to provide genus and species identification for each isolate.

Isolate S1.2 and S1.3 were 99% similar to *Pseudomonas* sp., while isolate WK2.4, WK2.6, and WK2.3 were similar to *Vibrio* sp.. This results were supported by microscopic examination which showed that all isolates

were Gram negative, rod shape for S1.2 and S1.4, and curved rod for WK2.3, WK2.4, and WK2.6. The characteristics and identities of isolates from waterfall and marine environment are presented in Table 4.

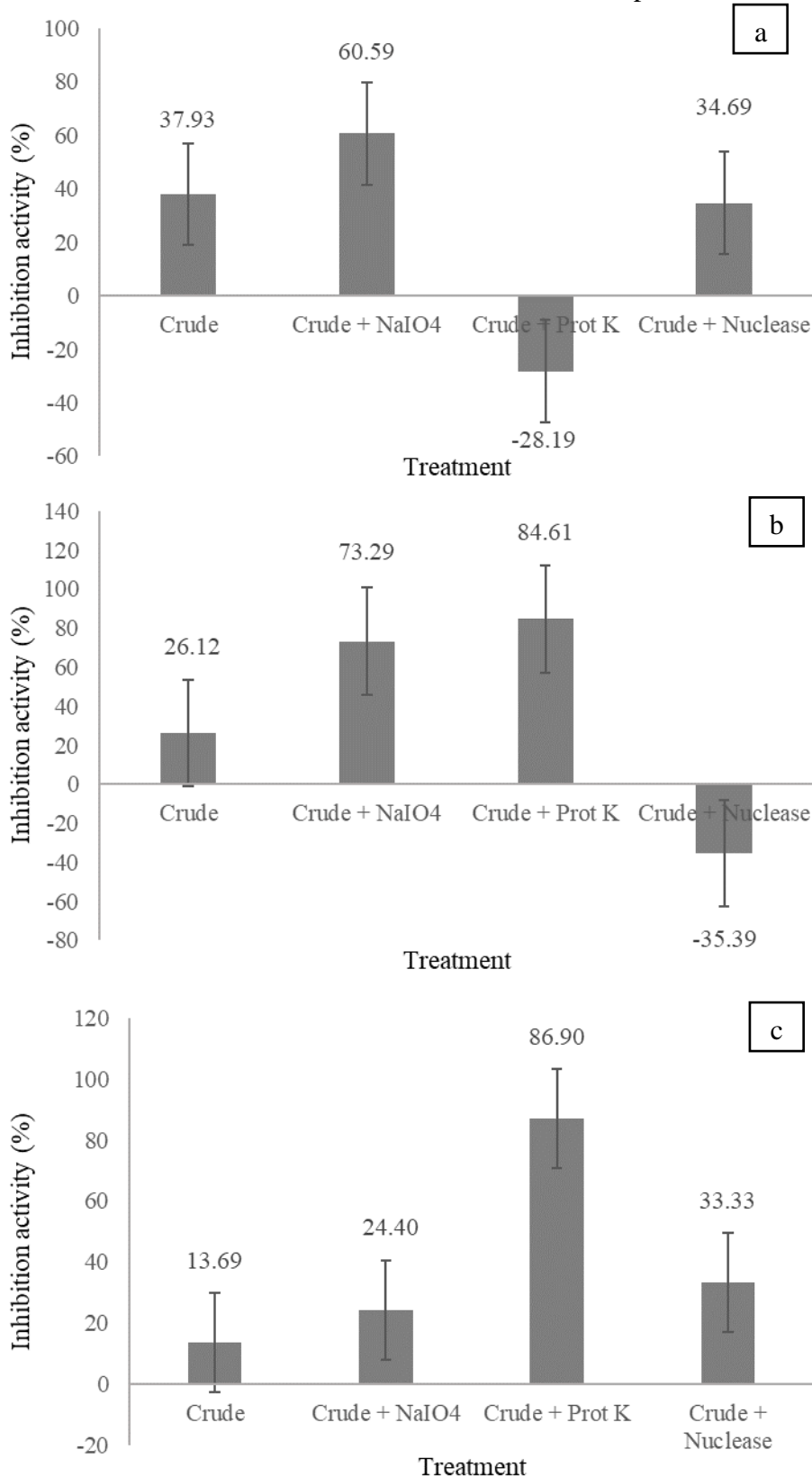


Figure 3. Effect of NaIO₄, proteinase-K, and nuclease on the antibiofilm activity of WK2.6 against tested bacterial biofilms. (a) *A. baumannii*, (b) *B. cepacia*, (c) *S. enterica*

Table 3. Bioactive compounds characterization. *P* Polysaccharide, *R* protein, *N* nucleic acid

Isolate	<i>A. baumannii</i>			<i>B. cepacia</i>			<i>E. coli</i>			<i>P. aeruginosa</i>			<i>S. enterica</i>			<i>S. aureus</i>		
	P	R	N	P	R	N	P	R	N	P	R	N	P	R	N	P	R	N
S1.2	-	+	+	[Black box]			[Black box]			-	+	+	+	-	+	-	+	-
S1.3	-	+	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+
WK2.4	+	+	+	-	+	+	-	-	+	-	+	-	+	-	+	-	+	-
WK2.6	-	+	+	-	-	+	+	+	+	-	+	+	-	-	-	[Black box]		
WK2.3	-	+	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	-

Legend: Black boxes = no biofilm activity; (+) = present; (-) = absent

Table 4. The profile of properties of isolates

Profile	S1.2	S1.3	WK2.4	WK2.6	WK2.3
Gram	-	-	-	-	-
Shape	Rod	Rod	Curved rod	Curved rod	Curved rod
Glucose	-	-	+	+	+
Lactose	-	-	-	-	-
Maltose	-	-	+	+	+
Mannitol	-	-	+	+	+
TSIA (slant/butt)	K/K	K/K	A/A	A/A	A/A
Citrate	+	+	-	-	-
Catalase	+	+	+	+	+
Growth in NaCl					
0%	Not tested	Not tested	-	-	-
1%	Not tested	Not tested	+	+	+
3%	Not tested	Not tested	+	+	+
6%	Not tested	Not tested	+	+	+
16S rRNA gene identity	<i>Pseudomonas</i> sp./ 99%	<i>Pseudomonas</i> sp./ 99%	<i>Vibrio</i> sp./ 99%	<i>Vibrio</i> sp./ 99%	<i>Vibrio</i> sp./ 99%
Accession number	KU872056.1	KU872057.1	KU872059.1	KU872060.1	KU872058.1

Legend: K = Alkaline; A = acid

DISCUSSION

Antimicrobial Activity

Many bacteria use quorum sensing to activate a number of traits including virulence factors involved in biofilm formation (Li & Tian, 2012). Nevertheless, the expression of virulence gene could be negatively affected by quorum sensing as reported in *Vibrio cholerae* (Lin, Kovacicova, & Skorupski, 2007). In the present study, quorum sensing and quorum quenching bacteria were evaluated for their antibiofilm activity. Antimicrobial compounds could reduce bacterial cell density and influence the biofilm development (Manavathu, Vager, & Vazquez, 2014), therefore it is important to assess the antimicrobial activity from the very beginning. The result of antimicrobial assay showed that neither bacteria from waterfall nor marine environment had this activity, hence they could be subjected to further experiments. Those

isolates might not produce chemical substances that might inhibit bacterial growth. Negative antimicrobial activity is an important character due to quorum sensing inhibition targets on bacterial signaling and is not focused on antibacterial activity (Choo, Rukayadi, & Hwang, 2006; Wang et al., 2019).

Quorum Sensing and Quorum Quenching Bacteria

Based on the first screening result, bacteria from waterfall and marine environment were able to produce Acyl-homoserine lactone (AHL), and consequently *C. violaceum* CV026 produced violacein (Figure 1, Figure 2). This result is in agreement with previous studies confirmed that bacteria isolated from aquatic environment have quorum sensing activity (Cuadrado-Silva, Castellanos, Arevalo-Ferro, & Osorno, 2013; Yunos et al., 2014). Among the five

characterized bacteria, three of them (WK2.3, WK2.4 and WK2.6) showed quorum sensing activity and belonged to the genus *Vibrio*. *Vibrios* are well known as quorum sensing bacteria commonly found in marine ecosystem and employ this communication system to regulate the expression of genes involved in biofilm formation, motility, bioluminescence, and virulence (Liu et al., 2013).

Quorum quenching bacteria in this study were detected by their ability to inhibit the AHL-dependent violacein production in *C. violaceum*. It was found from the second screening result that bacteria from two different origins, including WK2.3 displayed quorum quenching activity. This finding adds another report about the presence of quorum quenching bacteria in aquatic environment (Romero, Martin-Cuadrado, Roca-Rivada, Cabello, & Otero, 2011; Ghani et al., 2014). Furthermore, all bacteria did not show growth inhibition effect on *C. violaceum*. This proved that quorum quenching, also known as an antipathogenic approach, interferes with bacterial signaling without affecting their growth (Dong, Wang, & Zhang, 2007). The two remaining characterized bacteria (S1.2 and S1.3) were identified as members of genus *Pseudomonas*. This genus commonly found in many environments, such as soil, fresh water, and marine environments (Uğur, Ceylan, & Aslim, 2012). It has been reported that *Pseudomonas aeruginosa* PAO1 has pvdQ and quiP genes encoding quorum quenching AHL acylases (Wahjudi et al., 2011).

Although genus *Pseudomonas* has been widely studied for its quorum sensing system (Lee & Zhang, 2015; Rasamiravaka et al., 2015), the first screening result of isolates S1.2 and S1.3 seems to contradict previous studies. *P. aeruginosa*, for instance, is notably known to have two major and minor AHLs, N-butanoyl-L-homoserine lactone (BHL) and N-3-(oxododecanoyl)-L-homoserine lactone (OdDHL), N-hexanoyl-L-homoserine lactone (HHL) and N-3-(oxohexanoyl)-L-homoserine lactone (OHHL), respectively. An imbalance between levels of BHL and OdDHL, such as higher portion of long-chain AHLs (C10-C14), can inhibit violacein production. Insufficient BHL due to a deficiency in the Rhl system, i.e. one of its quorum sensing systems, may also

be considered as another possible reason for a negative screening result (Boşgelmez-Tinaz et al., 2005).

WK2.3 is particularly a marine bacterium which exhibited both quorum sensing and quorum quenching activities and were later identified as *Vibrio* sp. Further investigation is required to elucidate its mechanism. The ability of this isolate to inhibit quorum sensing is most likely related to antibiofilm effect of *Vibrio* sp.. In a previous study, Waturangi et al. (2011) reported the biofilm inhibition activity of *Vibriogallicus* supernatant against *V. cholerae*.

Inhibition of Initial Biofilm Development

The focus of attention in this study was antibiofilm activity of bacteria from waterfall and marine environment against *A. baumannii*, *B. cepacia*, *E. coli*, *P. aeruginosa*, *S. enterica*, and *S. aureus*. All crude extracts demonstrated biofilm inhibition in one pathogen, at least (Table 2). This result indicates that antibiofilm compounds produced by these bacteria are able to inhibit initial biofilm formation. The first essential step in biofilm formation is bacterial adhesion. The adherence is influenced by interactions between specific molecular groups, for example cell adhesion of *E. coli* is mediated by type 1 fimbrial adhesin FimH which binds to mannose as its receptor (Tchesnokova et al., 2011). Physical forces such as Van Der Waals forces, electrostatic interaction, hydrogen bonding, and Brownian motion forces also affect adhesion process (Busscher & Henny, 2012). Antibiofilm compounds can inhibit early biofilm formation in many ways, namely preventing cell adhesion by surface modification (Abdel-Aziz & Aeron, 2014) and interfering bacterial communication (Dong et al., 2007).

Characterization of Bioactive Compounds

In this study, antibiofilm compounds were differentiated into three types of components, namely polysaccharides, proteins, and nucleic acids. The mechanisms underlying their antibiofilm activities have been reported in many studies (Berne, Kysela, & Brun, 2010; Jorge, Lourenço, & Pereira, 2012; Chambers & Sauer, 2013; Rendueles, Kaplan, & Ghigo, 2013; Roy, Tiwari, Donelli, & Tiwari, 2018). Antibiofilm polysaccharides, protein, and

nucleic acid demonstrate the different modes of action in inhibiting biofilm formation (Roy et al., 2018). Polysaccharides inhibit biofilm formation by dispersing biofilm formation (Rendueles et al., 2013; Roy et al., 2018). Nucleic acids, either extracellular DNA or sRNA, can inhibit biofilm formation by preventing planktonic cells from adhering to the surface (Chambers & Sauer, 2013; Berne, Ellison, Ducret, & Brun, 2018). Meanwhile, protein such as antimicrobial peptide, could alter membrane potential and permeabilization, cell division and survival besides disaggregating lipopolysaccharide. Polysaccharide lyase and glycoside hydrolase enzymes can disperse the extracellular polysaccharide substance of biofilm. DNase I is able to digest extracellular DNA within the biofilm structure (Roy et al., 2018).

The characterization result obtained implies that the same bacteria produce different antibiofilm compounds against different pathogens. This is in agreement with previous research (Papa et al., 2015). Moreover, even bacteria within the same bacterial genus do not resemble either the spectrum antibiofilm activity or the compounds responsible for its activity, suggesting that bacteria from waterfall and marine environment inhibit biofilm formation specifically. None of the described compounds were present in the crude WK2.6 against *S. enterica*, indicating the involvement of other compounds. Aside from polysaccharides, protein, and nucleic acid, other antibiofilm compounds have been discovered such as fatty acid, rhamnolipids, and sophorolipids (Banat, De Rienzo, & Quinn, 2014; Rajalakshmi, Srinivasan, Poffe, Suresh, & Priyadarisini, 2014). Increases in inhibition activity of extract following the addition of NaIO₄, proteinase-K, or nuclease were observed. A study showed that the type of enzymes and their concentrations can influence the bacterial adhesion in two ways, preventing or stimulating the adhesion (Leroy, Delbarre, Ghillebaert, Compere, & Combes, 2008). It is suggested that the NaIO₄ compound or enzymes might affect the bacterial adhesion, however further research is required to identify the exact nature of the effects.

This study is by no means the first to find the antibiofilm activity of genus *Pseudomonas*. Previous study showed that exopolysaccharide EPS273 secreted by *Pseudomonas stutzeri* 273 has succeeded in inhibiting biofilm formation and dispersing preformed biofilm of *P. aeruginosa* PAO1. The primary components of its exopolysaccharide are glucosamine, rhamnose, glucose, and mannose (Wu, Liu, Jin, Xiu, & Sun, 2016). It has also been reported that genus *Vibrio* is known to exhibit antibiofilm activity. Exopolysaccharide A101 of *Vibrio* sp. QY101 prevents biofilm formation in a wide range of Gram positive and Gram negative bacteria and it primarily consists of galacturonic acid, glucuronic acid, rhamnose, and glucosamine (Jiang et al., 2011).

CONCLUSION

The present study shows antibiofilm activity of bacteria isolated from aquatic environment, that is waterfall and marine environment in Indonesia against a wide range of pathogenic bacteria. Among these indigenous aquatic bacteria, the bacterial genus identified were *Pseudomonas* and *Vibrio*. The composition of antibiofilm compounds derived from such bacteria may include but is not limited to polysaccharides, proteins, and nucleic acids. This study also reported the existence of quorum quenching activity in *Vibrio* species for the first time. Our promising results signify the potential of aquatic bacteria to be used for medical applications. Hence, further study is needed to screen inhibition activity of other pathogenic microorganism associated with biofilm.

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