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Evaluation of the potentials of *Bacillus subtilis* KM16 and *Pseudomonas sp.* PAP 26 isolated from the hot spring and crater lakes as antibiofilm agents

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Abstract

Bacteria can interact with each or other microorganisms by releasing, sensing, and reacting to small diffusible chemical signal molecules to alter their community behavior. This process, termed quorum sensing, is influenced by density of other bacteria that present in the environment. One example as a result of this process is the formation of biofilm. Biofilm consists of bacterial communities that attach to a surface and envelope themselves in secreted polymers. This formation can be beneficial to pathogenic bacteria because they become highly resistant to antibiotics and human immunity. Thus, antibiofilm agents that can inhibit biofilm formation are needed. The objective of this study were to screen and evaluate bacteria from hot spring and crater lakes that have antibiofilm activity against pathogenic bacteria. In this study, 26 isolates were successfully obtained and tested for quorum sensing and quorum quenching activities. Based on the result, two isolates, which were KM16 and PAP26, were found to have quorum quenching activity. Further research showed that KM16 and PAP26 had antibiofilm activity against more than six pathogenic bacteria. From characterization of the bioactive compounds, it is known that different compound from KM16 and PAP26 have different activity against each pathogen. In molecular identification, isolates KM16 and PAP26 were identified as *Bacillus subtilis* and *Pseudomonas sp.* through molecular identification.

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Introduction

Biofilm is microbial community embedded on inert or living surface and encaged in self-produced extrapolymeric substances (EPS) that contains proteins, polysaccharides, and extracellular DNA. The EPS provided structural strength and defense against environmental condition, host immunity, and antimicrobial agent (Davies, 2003; Singh *et al*., 2017). Most chronic infection is caused by biofilm-forming microorganisms and it is very difficult to eradicate them by only using conventional antibiotics or other antimicrobial therapy. In

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biomedical, biofilm can be formed on inanimate surface such as medical device, catheters, and living-tissue associated infection. Human immune system cannot correctly kill pathogenic bacteria or fungal and therefore, they may cause damage to encircling tissue (Taraszkiewicz *et al*., 2013). Biofilm can also contaminate food processing and attach to water pipe, henceit may cause food-borne and water-borne disease (Kokare *et al*., 2009).

Several strategies that can be used to inhibit biofilm formation are to interfere with quorum sensing mechanism and using antibiofilm agents. Quorum sensing can influence gene expression that is responsible for biofilm formation, virulence, sporulation, and pathogenicity. This process, alters wide-scale behavior of population in response to cell density. Therefore, mechanism that can interfere with quorum sensing mechanism, quorum quenching, is needed (Brackman & Coenye, 2015).

Thermophilic bacteria are a type of bacteria that can survive at high temperature environment, as in hot spring and crater lake. These bacteria have tremendously gained popularity in pharmaceutical and many industries because they can produce heat-stable bioactive molecule, for example, thermostable protease that does not denature at high temperature, but remains active at such temperature (Panda *et al*., 2013). Furthermore, antibiofilm activity of bacteria from hot spring and crater lakes in Indonesia has not been much explored. The purpose of this study was to screen and evaluate bacteria from hot spring and crater lakes that have antibiofilm activity against pathogenic bacteria.

Materials and Methods

Water Samples and Isolation of Bacteria

Water samples were obtained from hot spring and crater lakes at Mount Pancar, Bogor, Indonesia (Table 1). The media used for isolation were Luria Broth (LB) (10g tryptone, 5 g yeast extract, and 10 g NaCl, and 1000 mL ddH_2O) and Luria Agar (LA) (LB with 1.5% (w/v) bacteriological agar). A total 5 mL water sample was transferred into 250 ml conical flask containing 45 ml LB and incubated at 37°C, 70°C, and 80°C for 5 hours. The suspension was plated onto LA plates and incubated at 37°C overnight. Morphologically different colony that grew on LA plate was collected and inoculated by repeated streaking on the same medium. For short term preservation, isolates were streaked on LA and stored at 4°C. For long-term preservation, the culture was stored at -80°C in 15% (v/v) glycerol.

Table 1. Water source and condition

Source	Location	Condition (temperature; pH)
Hot Spring	Mount Pancar, Bogor, Indonesia	-
Merah Crater Lake Hitam Crater Lake Natural Crater Lake	Mount Pancar, Bogor, Indonesia	67°C; 7 48°C; 7 43°C; 7

Quorum Sensing Assay

Quorum sensing activity from bacteria sample was determined via Cross-feeding assay as sketched by Magdalena *et al.* (2020). N-acyl-homoserine lactone production was examined by streaking the isolate in parallel with a lane of the monitor strains *Chromobacterium violaceum* 026 (CV026) onto Brain Heart Infusion Agar (BHIA). BHIA medium incubated at 28°C for 48 hours. CV026 that showed production of violacein (purple

color) indicated a positive result of quorum sensing activity. Experiments were performed in triplicates.

Quorum Quenching Assay

The procedure used in this quorum quenching assay was agar well diffusion method which is a modification of the procedure of Soundari *et al.* (2014). Each isolate was grown in LB at 37°C until they reached absorbance value of 0.132 at λ=600 nm (McFarland 0.5). The inoculated LB was centrifuged twice at 12,000×g for 10 minutes and then filtered (0.22 µm) to obtain the cell-free supernatant. *C. violaceum* was grown in Brain Heart Infusion Broth (BHIB) at 28°C until it reached absorbance value of 0.5 at λ=600 nm (McFarland 0.5). One hundred µL of *C. violaceum* was spotted and streaked on BHIA using sterile cotton bud. Each well was made using cork borer. A total of 15 µL of cell-free supernatant was loaded into the well. LB was used as negative controls. The plates were incubated 28°C overnight and inhibition of purple pigment production was interpreted as positive results. Experiments were performed in triplicates.

Biofilm Inhibition Assay

The method used in this biofilm inhibition assay was the static biofilm assay which is a modification of the procedure of Magdalena *et al.* (2020). In this method, the biofilm inhibition was observed using 5% (v/v) concentration of cell-free supernatant. Pathogenic bacteria that were used in this assay were *Acinetobacter baumanii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 1637, *Escherichia coli* ATCC 4157, *Salmonella enterica* ATCC 51741, *Staphylococcus aureus* ATCC 25923, *Bacillus cepacia* ATCC 25416, and *Bacillus licheniformis* ATCC 12759. The test bacteria were grown in LB at 37°C until they reached absorbance value of 0.132 at λ=600 nm (McFarland 0.5).

In 96-well round bottom (U) microplates, 200 µL of tested bacteria were inoculated in each well along with 5% (v/v) cell-free supernatant. In this assay, 200 µL tested bacteria alone and 200 µL of uninoculated LB were used as negative control. The microplate was incubated at 37°C overnight. After incubation, the media and planktonic cells were discarded and the wells were rinsed twice using sterile distilled water and air-dried. Then, each well was stained with 200 µL of crystal violet and incubated for 30 minutes. The crystal violet was discarded and the wells were rinsed three times using sterile distilled water and air-dried. Afterwards, 200 µL of absolute ethanol was added to each well and incubated for another 30 minutes. Finally, the dissolved crystal violet was transferred to a new microplate and measured at λ=595 nm with Biorad 680 Microplate Reader. The percentage of biofilm inhibition was calculated using the following formula from Nikolić *et al.* (2014):

$$\%\ \text{biofilm inhibition} = \frac{(\text{OD growth control} - \text{OD sample})}{\text{OD growth control}} \times 100$$

Characterization of Bioactive Compounds

This method was adapted from the procedure of Jiang *et al.* (2011). Cell-free supernatant of each isolate was treated with proteinase-K (1 mg/ml), nuclease (100 µg/ml DNAse and 25 µg/mL RNAse), and $NaIO_4$ (20 mM) separately and incubated in 37°C for 12 hour. After incubation, treated cell-free supernatants were then used in static biofilm inhibition assay with cell-free supernatant concentration of 5% (v/v).

Microscopic Observation and Biochemistry Tests

For the microscopic observation the isolates were stained using Gram staining and observed under the magnification of 100x10. The biochemistry tests used for this research were catalase test, triple sugar iron agar (TSIA) test, carbohydrate fermentation test (glucose, lactose, maltose, and mannitol), and citrate test.

Molecular Identification of KM16 And PAP26

Genomic DNA was isolated using Wizard® Genomic DNA Purification Kit (Promega, Wisconsin, USA) and used as a DNA template in PCR. The identification of the isolates was conducted by amplifying the 16S rRNA gene using universal primer sequences 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAA GGC-3') (Marchesi *et al.*, 1998). The reaction mixture consisted of 0.5 µL DNA template, 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. Thermal cycling of 30-cycle PCR, included pre-denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and followed by a post-extension at 72°C for 20 minutes.

PCR products were then analyzed by 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 (1st BASE), and recorded with Gel Doc instrument (BioRad, USA). The marker used was 100 bp DNA ladder (Promega). PCR results were sent to 1st BASE, Malaysia for sequence. The sequences were used to identify the isolates using BLAST (NCBI) and submitted to GenBank.

Results And Disscussion

Isolation of Bacteria

In this research, water samples were collected from different crater lakes and hot spring at Mount Pancar, Bogor, Indonesia, using sterile glass container. The container then kept inside vacuum flask to maintain the water temperature. Bacteria were tested using broth media at diverse temperatures, but mostly at high temperature. In high temperature environment, most bacteria are believed to have characteristics of thermophile. This is because thermophilic bacteria have optimum growth temperature in the range of 45 - 80°C. Thermophilic bacteria are known to produce bioactive compound that can worked at high temperature (Taylor & Vaisman, 2010), such as antimicrobial agent and quorum sensing inhibitor is produced by hot spring cyanobacterial mats (Dobretsov, 2010).

In this study, 26 bacterial isolates were retrieved. They were 8 isolates from Merah crater lake, 12 isolates from Hitam crater lake, 3 isolates from Natural crater lake, and 3 isolates from hot spring (Table 2). All isolates were tested for quorum sensing activity via cross feeding assay using *C.violaceum* 026 (CV026) as a detector.

Table 2. Isolates from Mount Pancar, Bogor, Indonesia

Source	Number of Isolates	Code of Isolates
Merah crater lake	8	KM - 2, 7, 13, 14, 15, 16, 19, 22
Hitam crater lake	12	KH - 3, 5, 6, 8, 9, 10, 11, 17, 18, 20, 21, 23
Natural crater lake	3	KN - 1, 4, 12
Hot spring	3	PAP - 24, 25, 26

Quorum Sensing Activity

AHL-mediated quorum sensing control genes were responsible for colonization in eukaryotes in most bacteria species (Anbazhagan *et al.,* 2012). CV026 is AHL negative mutant because the presence of mini-Tn5 mutagenesis in CviI (AHL synthase), hence it requires exogenous AHL to produce violacein (purple pigment) (Vasavi *et al*., 2013). Based on the result, none of the CV026 produced purple pigment when they were grown together with each isolates. CV026 detected wide range of AHL with N-acyl side chains ranging from C_4 to C_8 in length, but did not detect AHL with N-acyl side chain raging from C_{10} to C_{14} (Anbazhagan *et al.,* 2012). This showed that all 26 isolates did not produce short chain AHL molecules. In spite of that, it did not rule out the possibility that these isolates can perform cell-to-cell communication. For example, wild type *B. subtilis* uses ComQXPA and Rap-Hpr quorum sensing systems to coordinate sporulation and competence. As gram-positive bacteria, *B. subtilis* uses small peptide as signal molecule (Kalamara *et al*., 2018). Thus, it cannot be detected by CV026. Another example is *Pseudomonas* species, *P. aeruginosa,* which uses LasI/LasR and RhII/RhIR quorum sensing system to control biofilm and generate extracellular enzymes. Although *Pseudomonas* as gram-negative uses AHL as their molecule signal (Umesha & Shivakumar, 2013), PAP26 is likely to produce long chain AHL molecules.

Bacteria have differences in quorum sensing systems, including signal types, receptors, and signal transduction mechanisms) (Waters & Bassler, 2005). Although they are in the same Gram type of bacteria, there is specificity in terms of signal types and receptors structure, hence this can cause inhibition (quorum quenching) because homologous signal molecule interferes with signal binding to receptor and decrease receptor concentration (Dong *et al.,* 2007). For example, *Staphylococcus aureus* has been divided into four groups based on the interaction between molecule signal and its receptor. Each group produced homologous AIP and only activated response in the same group member, but inhibited other group response (Umesha & Shivakumar, 2013). Therefore, quorum sensing activity could be used to inhibit quorum sensing of other bacteria.

Quorum Quenching and Biofilm Inhibition Activity

It was found out that bacterial isolates KM16 (Figure 1) and PAP26 had quorum quenching activity. Quorum quenching activity can be achieved by inhibiting signal synthesis, degradation of the signal molecule, and preventing signal molecule binding to transcriptional factors (Grandclément *et al.*, 2016).

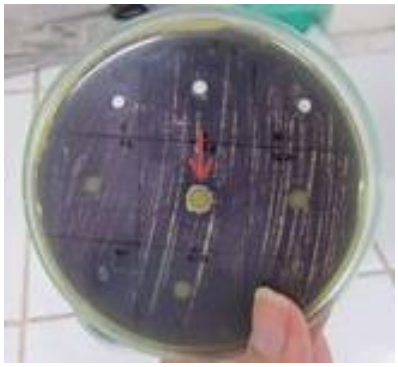

Figure 1. Positive result of the quorum quenching assay of KM16 isolate (red arrow)

The biofilm inhibition activity was performed using static biofilm inhibition assay to determine whether bacterial isolates KM16 and PAP26 can prevent biofilm formation of tested bacteria. Seven pathogen bacterial species were inoculated with 5% cell-free

supernatant (v/v) of KM16 and PAP26. Based on the result, bacterial isolates KM16 and PAP26 had antibiofilm activity towards several pathogenic bacteria (Table 3). KM16 showed the highest antibiofilm activity against *A. baumannii* with 82.29% activity. On the other hand, PAP26 showed the highest antibiofilm activity against *E. coli* with 84.09% activity. This activity can be influenced by polysaccharide, protein, or nucleic acid (DNA or RNA) compound in the isolate supernatant.

Table 3. Biofilm inhibition activity using 5% (v/v) crude extract of bacterial isolates KM16 and PAP26

Pathogenic bacteria	% inhibition	
	KM16	PAP26
A. baumannii ATCC 19606	82.29	78.74
P. aeruginosa ATCC 1637	48.28	36.72
S. aureus ATCC 25923	41.49	44.87
E. coli ATCC 4157	78.52	84.09
S. enterica ATCC 51741	29.13	34.72
B. cepacia ATCC 25416	14.77	-
B. licheniformis ATCC 12759	28.00	42.57

Characterization of Bioactive Compounds

Afterward, cell-free supernatant from each isolates was pre-treated using $NaIO_4$, proteinase-K, and nuclease. It is well known that proteinase-k, DNAse, and RNAse can degrade protein, DNA, and RNA. $NaIO_4$ is capable to hydrolyze carbohydrate molecules by oxidizing the carbons bearing hydroxyl groups and cleaving the C-C bonds (Jiang *et al.,* 2011).

In Figure 2(a), reduction of biofilm activity against *P. aeruginosa* was shown after cell-free supernatant of KM16 was pre-treated with proteinase-K and nuclease. On the contrary, cell-free supernatant pre-treated with $NaIO_4$ presented increasing activity. This can be caused by carbon and energy resources from breakdown of biopolymers used by pathogen to enhanced biofilm formation (Rabin *et al.,* 2015).

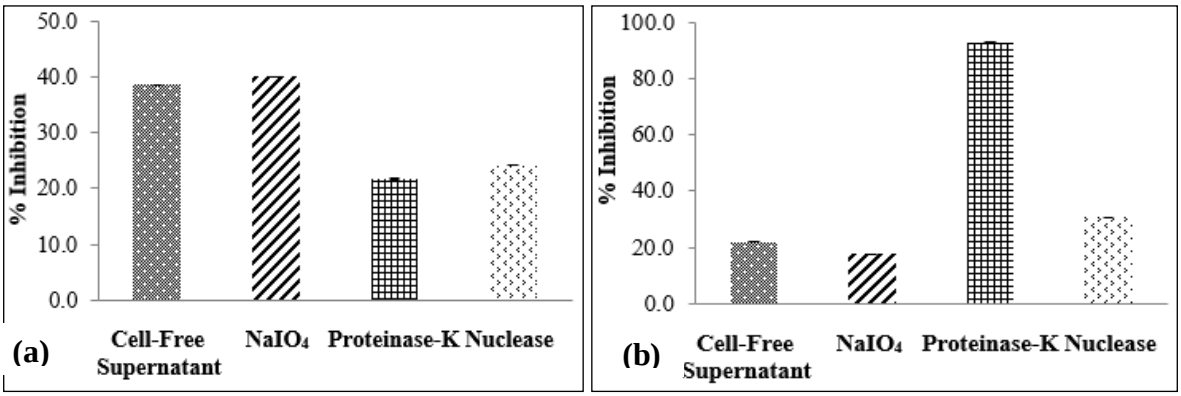

Figure 2. $NaIO_4$, proteinase-K, and nuclease effects on (a) KM16 crude extract against *P. aeruginosa*, (b) PAP26 crude extract against *S.aureus* biofilm.

In Figure 2(b), pre-treated cell-free supernatant of PAP26 with $NaIO_4$ exhibited decline in antibiofilm activity against *S. aureus*. This suggested that antibiofilm compound consisted of DNA, RNA, protein, and polysaccharide (Table 4). Biofilm matrix components in *P. aeruginosa* consist of Psl and Pel proteins that enhance intercellular adhesion and also

function as a barrier for immune and antibiotic attacks. Alginate plays role in structural stability and protection of biofilm. eDNA is created from random chromosomal DNA that serves as a cell-to-cell component united in the matrix biofilm. Protein and proteinaceous components serve as adhesion molecules and structural strength in biofilm formation (Karygianni *et al.*, 2020; Wei & Ma, 2013). Cell-free supernatant of KM16 produced protein and nucleic acid as antibiofilm agent, meanwhile PAP26 produce all three biomolecules.

S. aureus produce adhesion factor, such as serine-aspartate-repeat (Sdr) family, accumulation-associated protein (Aap), and Autolysin (Atl). Polysaccharide intercellular adhesion (PIA) or PNAG together with other polymer such as teichoic acids and proteins form major part of EPS in *Staphylococci*. Bap is also involved in intercellular adhesion and biofilm formation. Controlled cell death in *Staphylococci* released DNA that was needed for biofilm formation (Otto, 2008). KM16 produced polysaccharide and protein as antibiofilm agent. On the other hand, PAP26 produced all three biomolecules. Some bacteria exopolysaccharides can inhibit and destabilize biofilm from other bacteria without bacteriostatic and bactericidal activities, for example, *P. aeruginosa* cells degraded biofilm formation by *Staphylococcus epidermidis* and *S.aureus*. Therefore, Pel, Psl, and alginate do not only facilitate adhesive molecules to form biofilm, but also have antibiofilm properties (Rendueles *et al.,* 2013).

Table 4. Characterization of bioactive compounds of KM16 and PAP26 against seven tested bacteria

Pathogen		Bacterial Isolates	
		KM16	PAP26
A. baumannii	Polysaccharide	–	–
	Protein	√	√
	Nucleic acid	√	√
P. aeruginosa	Polysaccharide	–	√
	Protein	√	√
	Nucleic acid	√	√
S. aureus	Polysaccharide	√	√
	Protein	√	√
	Nucleic acid	–	√
E. coli	Polysaccharide	√	√
	Protein	–	√
	Nucleic acid	√	√
S. enterica	Polysaccharide	√	√
	Protein	√	–
	Nucleic acid	√	–
B. cepacia	Polysaccharide	√	■
	Protein	–	■
	Nucleic acid	√	■
B. licheniformis	Polysaccharide	√	–
	Protein	√	√
	Nucleic acid	√	√

*(√) = present, (-) absent, (■) not tested due to not having antibiofilm activity

There are three modes of action that are involved in polysaccharide antibiofilm activity, which are modifying abiotic and biotic surface properties, acting as signaling molecules that modulate gene expression, and acting as competitive inhibitor in carbohydrate-protein interaction. Biosurfactants change surface characteristic (wettability and charge of the surface), thus influencing interaction between bacteria and surface. Antibiofilm polysaccharides also alter physical properties of cell surface. For example, *B. licheniformis* reduced cell surface hydrophobicity, hence reducing *P. aeruginosa* colonization. Bacterial polysaccharides also caused down regulation of several genes that are responsible for biofilm formation, such us adhesion factor. This mechanism brings advantage to bacteria in bacteria competition and biofilm regulation (Rendueles *et al.,* 2013).

Protein acted as antibiofilm in the form of enzyme that degrade EPS matrix component and object that was trapped in EPS matrix. Negative charge of eDNA can act as repulsive force in initial attachment (Rabin *et al.,* 2015). eDNA also can bind to bacteria adhesive structure and inhibit cell attachment (Berne *et al.,* 2010). sRNAs can interfere translation process by binding to ribosome and promote mRNA degradation using RNase. sRNA can also terminate premature transcription by binding to a nascent mRNA (Mika & Hengge, 2013).

Identification of Bacteria

Bacterial isolate identification assay was performed by microscopy, biochemistry, and molecular assay (Table 5). Microscopy observation was done with Gram staining. From the result, each isolate had different gram type but similar morphology. Based on biochemistry assay, it was known that both isolates had completely different substrate preference.

Table 5. Bacterial isolate identification assay of KM16 and PAP26

Bacterial isolate identification assay			Isolate	
			KM16	PAP26
Microscopy	Gram		+	-
	Shape		Bacil	Bacil
Biochemistry	Citrat		-	+
	TSIA	Slant	Acid	Alkaline
		Butt	Acid	Alkaline
		Gas	-	-
		H_2S	-	-
	Glucose		+	-
	Lactose		-	-
	Maltose		+	-
	Mannitol		+	-
	Catalase		+	+
Molecular Identification	Genus		*B. subtilis*	*Pseudomonas* sp.
	Accession		KU877820.1	KU877821.1
	Identity		99%	99%

Bacterial identification with 16S rRNA showed that KM16 and PAP26 were identified as 99% *B. subtilis* and *Pseudomonas* sp. *B. subtilis* is gram positive and catalase positive

bacilli. *B. subtilis* can utilize citrate and produce acid from glucose, sucrose, maltose, and manitol fermentations. However, *B. subtilis* cannot produce acid from lactose (Saleh *et al.,* 2014). On the other hand, KM16 fermented lactose, but could not utilize citrate. Previous study showed that extracellular α-amylase from *B. subtilis* induced biofilm inhibition and degradation by disrupting exopolysaccharide in methicillin-resistant *Staphylococcus aureus* (MRSA) and *P. aeruginosa* (Kalpana *et al.,* 2012). Cyclic lipopeptide (LP) biosurfactants produced by *Bacillus*, also modified bacterial surface hydrophobicity and affected the development of flagella. Thus, it demonstrated significant anti-adhesive and antibiofilm activity (Moryl *et al.,* 2015)

Pseudomonas sp. is a gram negative, rod shaped, catalase positive bacteria, and naturally found in soil and water ecosystem. Most species do not require polysaccharide as carbon source. The genus *Pseudomonas* comprises species that are capable of living under diverse environmental conditions. *Pseudomonas* species are capable to biofilm formation and often resistant to antibiotics, disinfectants, detergents, heavy metals, and organic solvents (Rocha *et al*., 2019). The mechanisms of antibiofilm activity on *Pseudomonas* remain unclear.

Conclusions

Twenty six isolates were successfully isolated from crater lakes and hot spring at Mount Pancar, Bogor, Indonesia. From quorum sensing assay, it was found out that none of the isolates produced short chain AHL molecules. From quorum quenching and biofilm inhibition assay, bacterial isolates KM16 and PAP26 had quorum quenching and antibiofilm activities against tested bacteria. It was also known that different antibiofilm compound from each isolates could have different activity against each pathogen. KM16 and PAP26 isolates could be used as promising antibiofilm agent to treat bacteria biofilm infection. However, further research is needed to find out synergistic activity of this antibiofilm with antibiotic and to test whether biomolecule from KM16 and PAP26 is really thermostable.

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