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Potential Biofilm Inhibitor against *Pseudomonas aeruginosa* and *Bacillus cereus* from Crude Extract of Indonesian Coasts Bacteria

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Various research had been conducted to seek novel bioactive compounds from marine environment which are applicable toimprove health care. Treatments for *Pseudomonas aeruginosa* and *Bacillus cereus* biofilm has been one of the focus due to its effect in causing severe and persistence infection. Aside from abundance of antibiofilm screening, there have not yet novel antibiofilm discovered. Therefore, this research was conducted to explore potential antibiofilm compounds produced by bacteria originated from Indonesian coast. Bacteria were isolated from 22 coasts sediment samples from different coasts area across Indonesia. Crude extract from isolate were used for antibiofilm activity assay against *P.aeruginosa* and *B.cereus*. There were 45 isolates with antibiofilm properties in the crude extract. The three isolates with higher antibiofilm activity, specifically isolate A1.4, F1.10, and F4.5, were identified. Isolate A1.4, identified as *Psychobacter*, have both exopolysaccharide and nucleic acid as bioactive compounds in inhibition of *B.cereus* biofilm formation. Isolate F1.10 and F4.5 produced protein as bioactive compounds in crude extract which inhibit biofilm formation of *P.aeruginosa* and were identified as *Bacillus* and *Pseudomonas* respectively. Screening of potential bioactive compounds from crude extracts of bacteria isolated from Indonesian coasts showed promising antibiofilm activity against *P.aeruginosa* and *B.cereus*.

Key words: Coast sediment, antibiofilm, bioactive compounds, *Pseudomonas aeruginosa* & *Bacillus cereus.*

Biofilm aided pathogenic bacteria to survive from antibiotics therefore it is harder for patients to recuperate. Biofilm of *P.aeruginosa* is proven as the cause of nosocomial infections in hospital leading to harder treatment with antibiotics and associated with cystic fibrosis, pneumonia, and immunocompromised patients[1,2]. Infections of *B.cereus* causes evere food poisoning to human and is a major food contaminant. Biofilm formation of these pathogens instigate persistence infection and higher survival rate in the environment[3,4].Treatments to encounter biofilm formation of *P.aeruginosa* and *B.cereus* become a challenge to improve healthcare.

Wide range of bioactive compounds from marine bacteriahave been reported to exhibit potential as antibiofilm agent[1, 5-10]. These compoundswere produced by marine bacteria to interactin marine environment. The interaction might be antagonistic with each other, therefore producing compoundsmight enable to inhibit the growth of other bacteria[11-12]. Coasts area are found to have abundance microbial biofilm and unique interactions. The diversity in coasts microorganisms are due to its unstable environment throughout the day. Bacteria need to survive high salinity (10-32%), turbid water, unstable pressure from wind and waves, and solar radiation[12].

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Indonesian coasts area might serve as a potential source for bioactive compounds which exhibit antibiofilm activity. There have been many studies on antibiofilm compounds from marine environment. However, there are scarce information about the studies on marine bacteria in coast area in terms of antibiofilm compounds. Focus on this research took samples from Indonesian coasts area due to its diverse marine environment. This research was the first study on antibiofilm compounds produced by coast bacteria from Indonesian coasts.

MATERIALS AND METHODS

Samples collection

Samples collected from approximately 50 -100 cm below sea level surface sedimentin 22 coastal areas across Indonesianwerestored in 4°C.

Bacteria Isolation

Sediments were heated at 65°C for 60 minutes before inoculated in modified Starch Casein Broth medium (1g/100mL) for 7 days at 28°C. The modified SCB medium contains of 10g soluble starch, 0.3g casein, 1g K_2HPO_4, 2g KNO_3, 2g NaCl, 0.05g $MgSO_4$.7H2O, 0.02g $CaCO_3$, 0.01g KH_2PO_4 and 1 liter artificial seawater. Starch casein agar (SCA) were made by adding 15g bacteriological agar (Difco). Samples were diluted to 10^{-2} and spread to marine agar (MA, Difco) and SCA, and subsequently incubated for 5 days. Unique colonies were selected and cultivated in MA[13, 14].

Crude Extract Production

Isolates were inoculated in BHI medium (Oxoid) at 28ºC with 120 rpm agitation for 3 days[15]. After incubation, the medium were centrifuged (Thermo Scientific) at 13.684×g for 15 minutes. The supernatant was collected and transferred to a new microtube. Centrifugation were duplicatedto ensure all cells were pelleted. The cell-free supernatant were used as crude extract and stored at 4°C for a week or at -20ºC for a month[5].

Well Diffusion Assay

Antimicrobial activity assay used *P. aeruginosa* and *B. cereus* from Atma Jaya Culture Collection as test bacteria. About 100 µl test bacteria suspension with 0.5x McFarland (OD_{600}) concentration were streaked continuously on Mueller-Hilton Agar (MHA, Oxoid).Subsequently 50µl of crude extract were spotted into each well and incubated at 37ºC for 24 hours. The clear zone indicatedantimicrobial activity. The crude extract with antimicrobial activity were eliminated.

Biofilm Inhibition Activity Assay

Antibiofilm activity were perfomed using static biofilm assay. Test bacteria with 0.5x McFarland (OD_{600}) concentration were grown in Brain Heart Infusion Broth (BHIB) in 96 well polystyrene microplateincubated at 37ºC for 24 hours. Each well was added with 200 µl test bacteria and 20% (v/v) crude extract. Biofilmwere stained with 0.4% crystal violet for 10 minutes. Afterward, the crystal violet solution was discarded and rinsed with deionized water then air dried.Biofilm formation were quantified by measuring optical density of crystal violetat 595 nm absorbance using microplate reader (Biorad 680 Microplate Reader) after solubilized with 96% ethanol (5). As control, test bacteria were cultured without addition of crude extract.

Determination of bioactive compounds were done by treating crude extract of each isolate with Proteinase-K (Geneaid) (1 mg/ml), DNAseI (Sigma Aldrich) (100 mg/ml) + RNAseA(Sigma Aldrich) (25 mg/ml) or $NaIO_4$(Sigma Aldrich)(20 mM) and incubated at 37°C for 12 hours[7]prior to addition oftest bacteria. Antibiofilm activity were calculated in percentage by comparing the biofilm formed after treatment against control samples.

Identification of 16S rRNA gene sequence

Isolates with determined bioactive compounds were identifiedwith 16S rRNA gene sequence. The 16S rRNA gene were extracted using boiling lysis method followed by polymerase chain reaction (PCR). The PCR conditions were predenaturation (94°C, 5 min.); denaturation (94°C, 30 sec.); annealing (55°C, 30 sec.); extention (72°C, 60 sec.); post-extention (72°C; 10 min.); hold (4°C) with 30 cycles. Master mixes contained universal primer 63F (5'-AGTGAGCCCTGAAAATCA TGGACT-3') (Sigma) and 1387R (52 -GGG CGG WGT GTA CAA GGC-32), GoTaq® Green Master Mix (Promega) and DNA template. PCR samples were visualized using electrophoresis (80V, 400A, 70 min.) in 1% agarose gel. Samples were sent to Genetika Science and sequencing of the 16S rRNA gene were done by 1st BASE Sequencing INT (Singapore). The sequence obtained were compared to sequences within NCBI database (http://blast.ncbi.nlm.nih.gov/) using BLASTN 2.2.29+ program.

RESULTS AND DISCUSSION

Samples obtained from 22 different coasts area in Indonesia, including Java, Sumatra, Sulawesi, Bali-Nusa Tenggara, and Kalimantan. The total number of isolates obtained were 141 isolates (Table 1). The cell-free supernatant with higher antibiofilm activity were selected for further research (Table 2). There were 45 isolates exhibiting antibiofilm activity against *P. aeruginosa* and or *B.cereus*.Six isolates able to inhibit *B.cereus* biofilm formation above the threshold and eight isolates against *P.aeruginosa* biofilm formation. Screening of antibiofilm activity from crude extract of Indonesian coast bacteria exhibit results suggesting thatthere was a high possibility to find novel antibiofilm producer from coast bacteria. Bacteria isolated from each different location displayed antibiofilm activity against *P.aeruginosa* or*B.cereus*, notably some isolates could inhibit both test bacteria.Prior findings showed various marine bacteria produce antibiofilmcompounds, such as polysaccharide compoundss from *Vibrio* sp. QY101[1, 5-10]. Antibiofilm against other bacteria were produced as survival mechanism to compete for nutrional resources in environment [16-17].

Interestingly, the bioactive compounds which have antibiofilm activity was part of the biofilm matrix. Biofilm consist of water and the extracellular polimeric substance (EPS). The EPS wasmainly formed by polysaccharides, protein, nucleic acids and lipid[9,17-18]. To determine the bioactive compounds from crude extract, isolates with higher and more stable activity were selected. Crude extract were treated with specific enzymes to degrade certain subtance in EPS produced by isolated bacteria. The crude extract from these isolates showed different bioactive compounds.

Biofilm formation of *B. cereus* were inhibited by 30±3% compared to control after treated withcell-free supernant from isolate A1.4, but loss the ability to inhibit biofilm formation after treatment with natrium peroxide and nuclease (Table 3).This suggest that the bioactive compounds found in the crude extract were both polysaccharide and nucleic acids. Although many reports showed extracellular polysaccharide have antibiofilm activity,there have not yet specific mechanism defined regarding polysaccharide mode of action in biofilm formation inhibition. Rendueles

Table 1. Total number of Isolates obtained

Region	Sample Code	Location Origin	Number of Isolates
Java	A1	PantaiCarita	7
	A2	PulauPari (near fish cages)	7
	A3	PulauPari (near coral reef)	7
	A4	PantaiIndrayanti (private)	5
	A5	PantaiIndrayanti (public)	3
	A6	PantaiAlam Indah	5
	A7	PantaiPangandaran	4
	A8	PulauBurung Indah	3
	A9	PulauKongsi	3
	A10	PantaiPasirPerawan	6
	A11	PantaiPasirPerawan (near mangrove forest)	7
Sumatra	B1	PantaiTanjungPendam	9
	B2	PanaiPasirPadi	7
	B3	PantaiMabay	5
	B4	PantaiLengkuas	4
Sulawesi	C1	PantaiAkarena	7
Kalimantan	D1	PantaiSingkawang	6
Bali-Nusa	F1	Pantai Bali	9
Tenggara	F2	PantaiKuta	3
	F3	Pantai Sindhu Sanur	23
	F4	PulauGili	3
	F5	PantaiTuban	8

Table 2. Biofilm formation (%) of test bacteria afteraddition of cell-free supernatant (20% v/v)

Test Bacteria	A1.4	A3.2	A3.5	A3.3	A4.7	A4.9	A5.5	A5.6	A6.2	A6.3	A6.4	A7.2
B. cereus	55.3	-	-	69.1	81.7	-	78.2	-	67.9	83.9	59.2	-
P. aeruginosa	72.9	65.7	84.6	-	82.3	68.0	-	98.1	93.5	-	-	66.7
	A8.4	A9.7	A10.2	A10.4	A10.6	B1.3	B1.4	B1.6	B3.5	B4.2	B4.4	B4.8
B. cereus	52.7	-	58.8	-	69.4	85.1	-	69.5	-	75.2	96.6	51.2
P. aeruginosa	77.2	98.8	90.4	87.1	87.4	-	65.2	89.4	68.1	74.1	90.1	90.1
	B4.10	C1.6	C1.7	C1.8	D1.6	D1.7	F1.2	F1.3	F1.4	F1.5	F1.8	F1.9
B. cereus	89.3	83.2	69.1	-	-	61.8	96.6	78.6	91.2	62.6	91.2	61.5
P. aeruginosa	61.6	-	-	74.2	66.7	80.3	75.3	-	74.4	78.3	59.1	-
	F1.10	F2.1	F3.2	F3.11	F3.14	F4.5	F5.3	F5.5	F5.6			
B. cereus	-	-	-	-	-	-	-	-	-			
P. aeruginosa	60.6	89.7	96.5	44.2	51.2	50.7	56.0	61.4	43.6			

Table 3. Determination of bioactive compounds in crude extracts from isolates against *B. cereus*

Isolate Code	Bioactive compound		
	Polysaccharide	Nucleic acid	Protein
A1.4	+	+	-
A6.4	+	+	+
A8.4	ND	ND	ND
B4.8	ND	ND	ND
D1.7	-	-	+
F1.9	-	-	+

*ND: not determined

et al. (2013)described three possible mechanisms involving polysaccharide as antibiofilm compound against other bacteria. First, polysaccharide functioned in alternating the cell surfaces and abiotic surfaces, made it harder for targeted bacteria to attach to the surfaces[7,17,19-20]. Another mechanism stated polysaccharide play a role in regulating biofilm formation gene expression as signaling molecule or competitive inhibitor in polysacharide-protein interactions[17].

The EPS of *B.cereus* consist of 35-40% of protein and mixed of polysaccharide, extracelular nucleic acid, and lipid. Proteins in *B.cereus* biofilm matrix playedan important role in the biofilm establishment[19]. Consistent with possible mechanism suggested before, polysaccharide from isolate A1.4 cell-free supernant might be able to alternate the cell or abiotic surface and to function as competitive inhibitor in polysaccharide-protein interactions. Additionally, studies reportedthe role of eDNA and sRNA asa regulatory system which prevent biofilm formation and initiating dispersion of biofilm[8-10]. Isolate A1.4, which was isolated from Carita Beach, were identified as *Psychobacter* (NCBI accession number KJ670157). Previous studies revealed the bacterium *Psychobacter* have bioactive compoundss which could act as antitumor, antifungal, or antibacterial[21].

Biofilm formation of *P.aeruginosa* were reduced to 82±4% compared to control by isolate F1.10 cell-free supernatant and 73±5% compared to control by isolate F4.5 cell-free supernatant. The crude extract from isolate F1.10loss the antibiofilm activity after treatment with proteinase-K and isolate F4.5 loss the antibiofilm activity after treatment with $NaIO_4$ and proteinase-K (Figure 1).This result demonstrated protein or peptides as bioactive compounds in cell-free supernatant from isolate F1.10 and F4.5. Mechanisms on protein or peptides as bioactive compounds include possibility of cell signaling interference, cell signal and biofilm matrix degradation[5,6, 22]. The EPS of *P.aeruginosa*were mostly polysaccharide, such as alginate, Pel and Psl[16, 17]. The presence of these polysaccharides explain the reason ofthe highly inhibition of biofilm formation (35±3%) after addition of sodium peroxide to *P.aeruginosa* culture. The protein or peptides in the crude extract coulddegrade the polysaccharide of *P.aeruginosa* biofilm matrix or cell signaling interference.

Isolate F1.10 was isolated from Bali Beach and identified as bacteria from the genus *Bacillus* (NCBI accession number KJ670159). Various antibiofilm properties from genus *Bacillus*had been reported[1, 20, 22]. Interestingly, isolate F4.5 which isolated from Gili Island, was identified as *Pseudomonas* sp (NCBI accession number

KJ670160). This suggest there were antagonistic interaction even in the same genera. Prior finding suggesting antibiofilm activity from *P. aeruginosa* against *Stapphylococcus epidermidis*[16]. There is also evidence in antagonistic interaction between different species of the same member in genus *Bacillus*[22].

Further studies needs to be conducted to analyze more deeply the diversity and potential novel bacteria found in Indonesian coasts area. We observed unstable inhibition activity and unidentified bioactive compoundsin cell-free supernatant, suggesting that bioactive compounds which have antibiofilm activity need

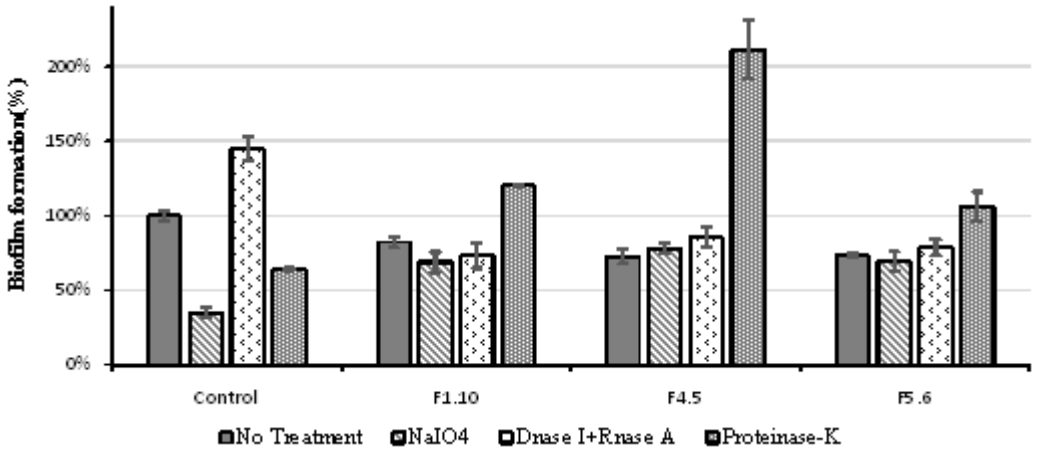

Fig. 1. Biofilm formation (%) of *P. aeruginosa* with addition of crude extract. Control did not added with crude extract. Crude extract treatment were done before addition to bacteria culture

to be purified and characterize to obtain exact data on its ability to inhibit biofilm formation. Along the line, there is need to analyze the possibility of lipid as antibiofilmcompounds and biofilm inhibition mechanisms for further application in medical field. Although there are still many research needed to be performed before application, the results presented in this study has encourage the potential of coast bacteria as novel antibiofilm producer.

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