Malaysian Journal of Microbiology, Vol 12(4) 2016, pp. 291-299
http://dx.doi.org/10.21161/mjm.80915

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Characterization of bioactive compound from actinomycetes for antibiofilm activity against Gram-negative and Gram-positive bacteria

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Received 11 January 2016; Received in revised form 16 May 2016; Accepted 29 June 2016

ABSTRACT

Aims: Biofilm is an assemblage of microorganisms enclosed in a matrix of extracellular materials, such as, extracellular polysaccharide (EPS), and relates to bacterial virulence, pathogenesis, and environmental survival. Bacteria inside biofilm are more resistant to conventional antibiotics and the host immune system. Non-biocidal antibiofilm compounds have been developed to address this problem. Specifically, actinomycetes have known to produce many metabolite compounds that have useful application in medicine and biotechnology. The study aimed to characterize bioactive compounds from actinomycetes crude extract that have capability as a multispecies antibiofilm agent.
Methodology and results: In this study, none of the isolates had shown any antimicrobial activity. Based on the antibiofilm assay, most of the isolates have the capability to inhibit and to destroy biofilm formation of pathogenic bacteria at a 5% and 10% dosage. The crude extracts showing the highest activity for antibiofilm inhibition were extracted from *Streptomyces* sp. The characterization of the bioactive compounds showed that different components of a particular isolate responsible for its antibiofilm activity against pathogenic bacteria. The SW19 isolate had a nucleic acid, KP12 isolate had a combination of the three component (polysaccharide, protein, and nucleic acid), and CW17 isolate had a combination of polysaccharide and nucleic acid as the active compound for antibiofilm activity.
Conclusion, significance and impact study: Thus, the bioactive crude extracts from actinomycetes has high potential to be used in treating biofilm-related infection and further research is needed to purify the bioactive compound from the crude extract which has antibiofilm activity against Gram-positive and Gram-negative pathogens.

Keywords: actinomycetes, bioactive compound, antibiofilm

INTRODUCTION

Biofilm is an assemblage of microorganisms enclosed in a matrix of extracellular materials such as extracellular polysaccharide (EPS), and relates to bacterial virulence, pathogenesis, and environmental survival. Formation of biofilm made cells more resistance to stress, antibiotics, host immune response, and disinfectant compared to planktonic bacteria (Jiang *et al.*, 2011).

Biofilms consist mainly of extracellular polysaccharide (EPS), protein, and extracellular nucleic acid (Carpenter, 1971). The development of biofilm can be seen as a five-stage process: initial reversible adsorption of cells to the solid surface, production of extracellular polymeric matrix substances resulting in an irreversible attachment, early development of biofilm architecture, maturation, and dispersion of single cells from the biofilm. A bacterial cell initiates attachment to the surface by using EPS glycocalyx polymers and this first step is important in biofilm formation. In Gram-negative bacteria, glycocalyx polymers also form a rigid structure called a capsule or loose slime layer. Some Gram-positive bacteria also have a capsule, but it is more common in Gram-negative bacteria (Vidyawan and Waturangi, 2013).

A majority of the biofilm formation involves cell-to-cell signaling using an autoinducer. This bacterial communication is called quorum sensing. There are several different quorum sensing autoinducers in bacteria. In Gram-negative bacteria the autoinducer compounds are acylatedhomoserine lactones (AHL/AI-1). In addition to AHL, 2-heptyl-3-hydroxy-4-quinolone (PQS) and diketopiperazines have also been detected in *Pseudomonas aeruginosa* and other bacteria (Pesci *et al.*, 1999). In Gram-positive bacteria, peptides are used as autoinducers. Autoinducer 2 (AI-2) was recently found in both Gram-positive and Gram-negative bacteria (Vendeville *et al.*, 2005).

Biofilm formation is also mediated by a number of mechanical, biochemical and genetical tools. The pili and flagella are generally involved as adhesive structures to help in attachment to biotic or abiotic surfaces. The role of an attachment factor, cellulose fiber and lipopolysaccharide (LPS) interactions to maintain strength and integrity in biofilm making in *Pseudomonas*

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

fluorescens SBW25 has also been studied. The requirement of type IV pili has been implicated in maximal biofilm formation by *Clostridium perfringens* (Varga *et al.*, 2008).

Using the protection provided by the biofilm, pathogenic bacteria can more easily cause chronic infection in their host. If the bacterial colonies are not immediately separated from the surface, they can anchor themselves more permanently using cell-adhesion molecules. Therefore, searching for compounds or strategies to decrease or destroy bacterial biofilm formation is essential.

One potential strategy that may be effective to overcome biofilm formation is by screening for bacteria that have the capability to produce metabolite compounds that can act as antibiofilm agents. Actinomycetes are Gram-positive bacteria that are widespread in nature and commonly produce many important sources of biologically active compounds such as antitumor agents, antibacterial compounds, and enzyme inhibitors. In a previous study, the screening of actinomycetes from various environment sediments such as lake, river, and agriculture areas in Indonesia resulted in ten potential isolates with antibiofilm activity against *V. cholerae* (Vidyawan and Waturangi, 2013).

Actinomycetes are Gram-positive bacteria that have a high percentage of guanine and cytocine in the DNA and are very widespread in nature. It is an intermediate form between bacteria and fungi. They have a unicellular mycelium shape which is the composition of branched long hyphae (Pandey *et al.*, 2008; Johnson *et al.*, 2012).

The diameter ranges from 0.5 to 0.8 µm. These myceliums can develop in the lower layer on the surface or bottom of bacteria colonies, and grow towering into the air like an antenna. The majority of actinomycetes are free living, saprophytic bacteria, widely distributed in soil, water and colonizing plants, and are even able to live in extreme conditions (Seong *et al.*, 2001; Debananda *et al.*, 2009; Moussa *et al.*, 2011; Nakade, 2012).

Of all actinomycetes bacteria, the genus *Streptomyces* is the most productive group of actinomycetes, because it can produce many antibiotics and other classes of biologically active secondary metabolites (Hozzein *et al.*, 2011). They cover around 80% of total antibiotic products, with other genera trailing numerically. *Micromospora* is the runner up with less than one-tenth as many as *Streptomyces* (Pandey *et al.*, 2008). Some examples of secondary metabolites (antibiotics) produced by the *Streptomyces* group includes erythromycin, tetracycline, streptomycin, chloramphenicol, neomycin, nystatin amphotricin, kanamycin and cycloheximide, which have an important role in the field of medicine (Hozzein *et al.*, 2011; Raja and Prabakarana, 2011).

Some reports have shown that actinomycetes have the capability to inhibit and reduce the biofilm formation of *V. Cholerae* (You *et al.*, 2007) and *Vibrio spp.* (Nithya and Pandian, 2010). *Streptomyces* sp. BFI 230 and *Kribella* sp. BFI 1562 also have been reported to have antibiofilm activity against a *P. aeruginosa* biofilm (Kim *et al.*, 2009). The present work aimed to study and characterize bioactive compounds from actinomycetes crude extract that have capability as a multispecies antibiofilm agent from ten isolates of actinomycetes and provide a new alternative treatment for biofilm-associated disease.

MATERIALS AND METHODS

Growth of actinomycetes

Ten actinomycetes isolates used in this study were obtained from a previous study that isolated these specimens from various environments, such as lakes, rivers, and a paddy field (Table 1). These isolates were cultured in Streptomyces agar (malt extract 10 g, yeast extract 4 g, agar 12 g, $CaCO_3$ 2 g, glucose 4 g and ddH_2O 1000 mL) at 28 °C for 7 days. The isolates were then transferred to tryptone soya broth (Oxoid, UK) for fermentation at 28 °C for 7 days. The fermentation broths were then centrifuged at 7,798 × g for 15 min and the supernatants were used as bioactive crude extract of the isolates for further assay.

Table 1: Isolates of actinomycetes.

No	Origin of Isolates	Name	Similarity with	Accession Number
1	Cunca Wulang River, West Flores	CW01	*Arthrobacter* sp.	JX434848
2	Cunca Wulang River, West Flores	CW17	*Streptomyces* sp.	JX434845
3	Kulon Progo River, Yogyakarta	KP04	*Streptomyces* sp.	JX434847
4	Kulon Progo River, Yogyakarta	KP12	*Streptomyces* sp.	JX434844
5	Kulon Progo River, Yogyakarta	KP17	*Streptomyces clavuligerus*	JX434846
6	Paddy Field, Gancahan 8 Village, Sleman	SW03	*Streptomyces* sp.	JX434841
7	Paddy Field, Gancahan 8 Village, Sleman	SW12	*Streptomyces carpaticus*	JX434849
8	Paddy Field, Gancahan 8 Village, Sleman	SW13	*Streptomyces* sp.	JX434850
9	Paddy Field, Gancahan 8 Village, Sleman	SW19	*Arthrobacter* sp.	JX434843
10	Telaga Biru Lake, Cibodas	TB12	*Arthrobacter mycorens*	JX434842

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Bacterial isolates and culture condition

The pathogenic bacteria used in this research were *P. aeruginosa* (ATCC 27853), Enterotoxigenic *Escherichia coli* (ETEC) (Atma Jaya Collection Culture), *Vibrio parahaemolytius* (ATCC 17802), *V. cholerae* (Atma Jaya Collection Culture), *Streptococcus pneumoniae* (ATCC 49616), *Staphylococcus aureus* (ATCC 29213), and *Enterococcus faecalis* (ATCC 33186). All pathogenic bacteria were grown on Nutrient Agar (Oxoid) at 37 °C overnight except for *V. parahaemolytius* and *V. cholerae*, which were grown on Thiosulfate Citrate Bile Salt (Oxoid) medium at 28 °C for 24-28 h.

Antimicrobial assay

Actinomycetes isolates were grown on Tryptone Soya Broth (Oxoid) and incubated at 28 °C for 7 days. The fermentation broth was then transferred to sterile polypropylene and centrifuged at 7,798 × g at 4 °C for 15 minutes using a 50 mL centrifuge (Sorvall® Legend RT ID-Nr:20057916). The supernatant was used as crude extract of the isolates and stored in sterile polypropylene (Extra Gene, Taiwan). The crude extracts obtained from the previous process were stored at 4 °C within one week or stored at −20 °C for months (Nithya and Pandian, 2010).

Screening of antimicrobial activity of the isolates was done using a Kirby-Bauer disc diffusion method. All of the pathogenic bacteria were uniformly streaked in three directions on the test plates of Mueller Hilton Agar (Oxoid, UK) with a sterile cotton swab. Sterile antibiotic discs (6 mm diameter) impregnated with the crude actinomycetes extract were transferred to the test plates and incubated at 37 °C for 24-48 h. The zone of inhibition in mm diameter, were read and taken as the activity against the test pathogenic bacteria. Chloramphenicol (Oxoid, UK) were used as positive control for *S. aureus*, tetracycline (Oxoid, UK) for ETEC, *P. aeruginosa*, *S. pneumoniae* and *E. faecalis*, while ciprofloxacin were used for *Vibrio* sp.

Biofilm inhibition and destruction assay

The pathogen bacteria were transferred from their medium agar plate into Brain Heart Infusion (BHI) broth (Oxoid) and incubated at 28 °C overnight for *V. cholerae* and *V. parahaemolyticus* or 37 °C for the others. A static biofilm assay was used in this research. The pathogenic bacteria were incubated in 96-well microtiter plates (polystyrene) containing 200 µL BHI medium with 1% glucose/well at their optimum temperature for 24 h. Planktonic cells and spent media were discarded, and adherent cells were gently rinsed twice with deionized water and allowed to air dry before being stained. The biofilms were stained by 200 µL 0.4% (w/v) crystal violet solution for 10 min, after which the dye was discarded and the wells were rinsed twice with deionized water. The wells were air dried before being solubilized of the crystal violet with 200 µL dimethyl sulfoxide (You *et al.*, 2007). The optical density was determined at 595 nm using a microplate reader (Biorad 680 Microplate Reader).

To screen for the destruction of mature biofilm, biofilms were developed in a 96-well microtiter plate in BHI medium with glucose to maximize biofilm formation and were incubated at optimum temperature for each pathogenic bacteria, overnight. Mature biofilms were incubated with actinomycetes supernatants at 5% and 10%, respectively, for 30 min at 37 °C and were assayed in 96-well polystyrene microplate as described above. To study biofilm inhibition, the biofilms were developed with actinomycetes supernatants in 96-well microtiter plate containing BHI medium with glucose and were incubated at 28 °C for 24 h (You *et al.*, 2007).

Characterization of actinomycetes

Selected actinomycetes supernatant were treated with proteinase K (1 mg/mL), DNase I (100 µg/mL) + RNase A (25 µg/mL), and $NaIO_4$ 20 mM at 37 °C for 12 h. These pre-treated supernatants were further used for biofilm inhibition and destruction assay (Jiang *et al.*, 2011).

RESULTS

Antimicrobial activity

Ten isolates of actinomycetes bacteria showed negative antimicrobial activity against all tested pathogens, while all antibiotics that were used as positive control inhibited bacterial growth. Thus, the activity of the antibiofilm was a result from non-biocidal antibiofilm crude extract.

Biofilm inhibition and destruction assay

To determine the spectrum of actinomycetes extracts as antibiofilm candidates, all crude extracts were tested against Gram-positive and Gram-negative tested bacteria. From the test results, all crude extracts were capable of inhibiting biofilm formation of pathogenic bacteria.

The results demonstrated that *Streptomyces* sp. (KP12, KP04 and CW17 isolates) and *Arthrobacter* sp. (SW 19 isolate) had the highest activity for inhibiting the pathogen biofilm. *P. aeruginosa* biofilm were inhibited by KP12 isolate, ETEC by KP04 isolate, *E. faecalis* and *V. parahaemolyticus* by CW17, *S. aureus* and *S. pneumoniae* by SW19 at the lowest concentration at 5% (v/v) (Table 2).

Destruction assay of pathogenic bacteria biofilm by treatment of actinomycetes crude extracts at concentrations of 5% (v/v) and 10% (v/v) are shown in Table 2. The results demonstrated that *Streptomyces* sp. (SW03, CW17, and SW12 isolates) and *Arthrobacter* sp. (TB12 and CW01 isolates) had the highest activity against the pathogen biofilm. *P. aeruginosa* and *V. parahaemolyticus* biofilm were destroyed by SW03 isolate, ETEC by CW01 isolate, *S. aureus* by CW17 isolate, *S. pneumoniae* by TB12 isolate, and SW12 by *E. faecalis* at the lowest concentration (5% v/v).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 2: Biofilm inhibition and destruction activity (%) of actinomycetes against Gram-positive and Gram-negative bacteria.

Pathogen[a]	[Crude Extracts]	Inhibition Activity (%)									
		SW12	SW13	SW19	CW17	KP17	TB12	CW01	KP04	KP12	SW03
SA	5%	57.23	46.89	60.92*	53.95	29.33	46.72	47.39	33.19	47.23	37.89
	10%	59.92	58.74	61.01	60.10	36.97	51.00	53.95	47.31	50.50	47.31
SP	5%	41.71	42.44	73.63*	53.67	71.27	65.88	75.51	66.01	71.01	68.62
	10%	68.81	54.88	74.30	67.44	76.37	66.80	76.66	75.61	71.84	70.79
EF	5%	52.26	74.76	15.49	85.80*	80.09	81.22	82.73	81.28	70.02	71.42
	10%	56.87	81.01	26.10	84.21	81.84	86.29	83.22	81.52	73.65	78.80
PA	5%	59.17	74.74	77.54	71.93	77.78	81.75	80.42	83.1	85.91*	62.14
	10%	66.78	79.82	75.12	80.98	79.54	83.91	84.05	88.86	90.59	68.01
ETEC	5%	87.23	64.67	66.81	57.36	66.36	70.53	71.96	90.56*	62.31	50.31
	10%	91.52	72.37	68.83	59.63	71.36	76.28	80.44	91.05	62.2	61.12
VP	5%	39.54	57.36	49.08	69.77*	67.08	14.14	22.74	61.08	47.37	69.58
	10%	44.31	59.53	57.34	72.12	67.56	27.82	41.86	65.26	69.29	65.97

Pathogen[a]	[Crude Extracts]	Destruction Activity (%)									
		SW12	SW13	SW19	CW17	KP17	TB12	CW01	KP04	KP12	SW03
SA	5%	69.16	47.65	52.44	69.41*	60.76	49.33	66.30	59.16	66.05	67.56
	10%	70.92	65.29	63.11	70.59	69.08	64.54	67.39	67.82	71.60	72.02
SP	5%	41.74	38.19	66.82	63.77	66.98	68.75*	59.68	58.14	61.15	50.12
	10%	56.02	51.27	67.40	64.62	70.68	69.71	67.55	59.99	62.27	52.08
EF	5%	78.36*	66.86	76.74	76.01	75.85	65.64	70.18	73.99	57.29	49.51
	10%	78.44	71.64	78.61	78.04	76.09	73.91	73.26	74.39	69.12	66.16
PA	5%	45.22	41.64	47.55	46.37	52.45	58.69	42.13	63.60	61.50	65.89*
	10%	55.73	83.97	58.25	51.27	53.89	63.28	65.45	70.16	63.66	76.94
ETEC	5%	41.05	62.68	43.43	52.06	55.68	51.19	63.74*	60.44	47.36	47.35
	10%	67.58	67.63	56.98	57.82	58.68	56.36	64.20	62.41	50.26	48.87
VP	5%	47.86	53.36	46.99	47.55	47.44	48.81	54.95	48.98	52.91	56.31*
	10%	73.73	55.12	50.17	57.51	54.04	57.51	57.25	50.85	52.56	54.78

SA, *S. aureus*; SP, *S. pneumoniae*; EF, *E. faecalis*; PA, *P. aeruginosa*; VP, *V. parahaemolyticus*
* The highest antibiofilm activity for each pathogenic bacteria.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Characterization of bioactive compound

The characterization was done by static inhibition assay using pre-treated crude extract. The pre-treatment using nuclease, proteinase-K and $NaIO_4$ could decrease the antibiofilm activity of a certain crude depends on the bioactive compounds involved in a specific antibiofilm activity. In Figure 1(F), only the pre-treatment of SW19 crude extract using nuclease shows a decrease in antibiofilm activity, while the pre-treatment using $NaIO_4$ and proteinase-K shows no effects in antibiofilm activity against *S. pneumonia* (Figure 1D, 1E). In Figure 2(A-C), all the pre-treatment of KP12 crude extract using $NaIO_4$, proteinase K, and nuclease shows a decrease in antibiofilm activity against *P. aeruginosa*. In Figure 2 (D and F), the pre-treatment of CW17 crude extract using $NaIO_4$ and nuclease shows a decrease in antibiofilm activity, while the pre-treatment using proteinase-K shows no effects in antibiofilm activity against *V. parahaemolyticus* (Figure 2E). Finally, the bioactive compound in SW19 crude extract was likely nucleic acid, KP12 crude extract was likely combination of three components (polysaccharide, protein, and nucleic acid), and CW17 crude extract were likely polysaccharide and nucleic acid.

In contrast to the other isolates, none of the three pre-treatments show any effect to the crude extracts (SW19, TB12, and KP04) against pathogenic bacteria (*S. aureus*, *E. faecalis*, and ETEC), respectively. Therefore, none of the three components (polysaccharides, proteins, or nucleic acids) was responsible for crude extract antibiofilm activity against pathogenic bacteria.

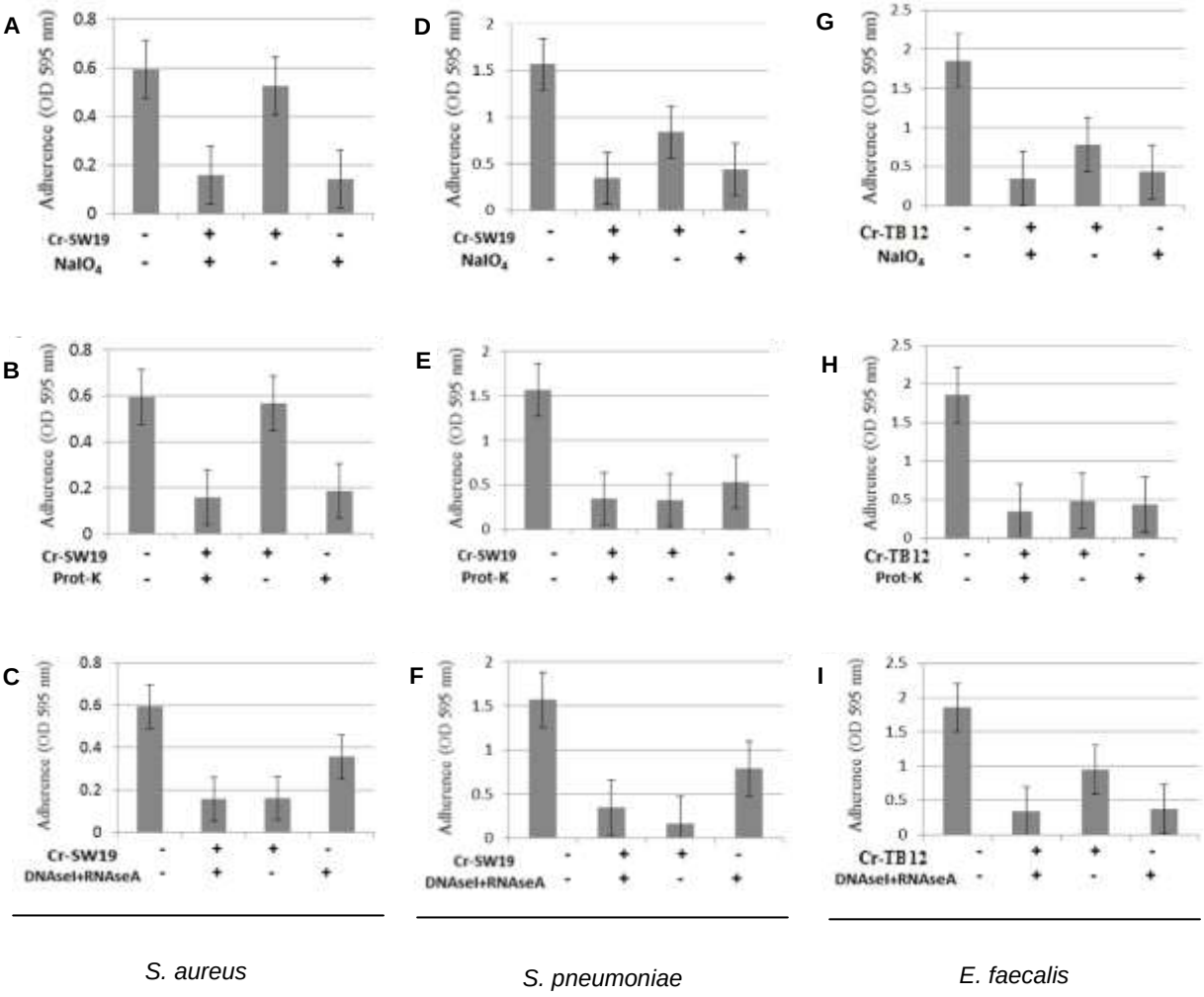

Figure 1: Effect of $NaIO_4$, proteinase K, and nuclease pre-treatment of actinomycetes crude extracts against Gram-positive pathogenic bacteria. Crude extract at 5% was respectively treated with $NaIO_4$ (20 mM), proteinase K (1 mg/mL), DNase I (100 mg/mL) + RNase A (25 mg/mL) at 37 °C for 12 h. Crude extract SW19 against *S. aureus* (A-C) crude extract SW19 against *S. pneumonia* (D-F), and crude extract TB12 against *E. faecalis* (G-I).

ISSN (print): 1823-8262, ISSN (online): 2231-7538

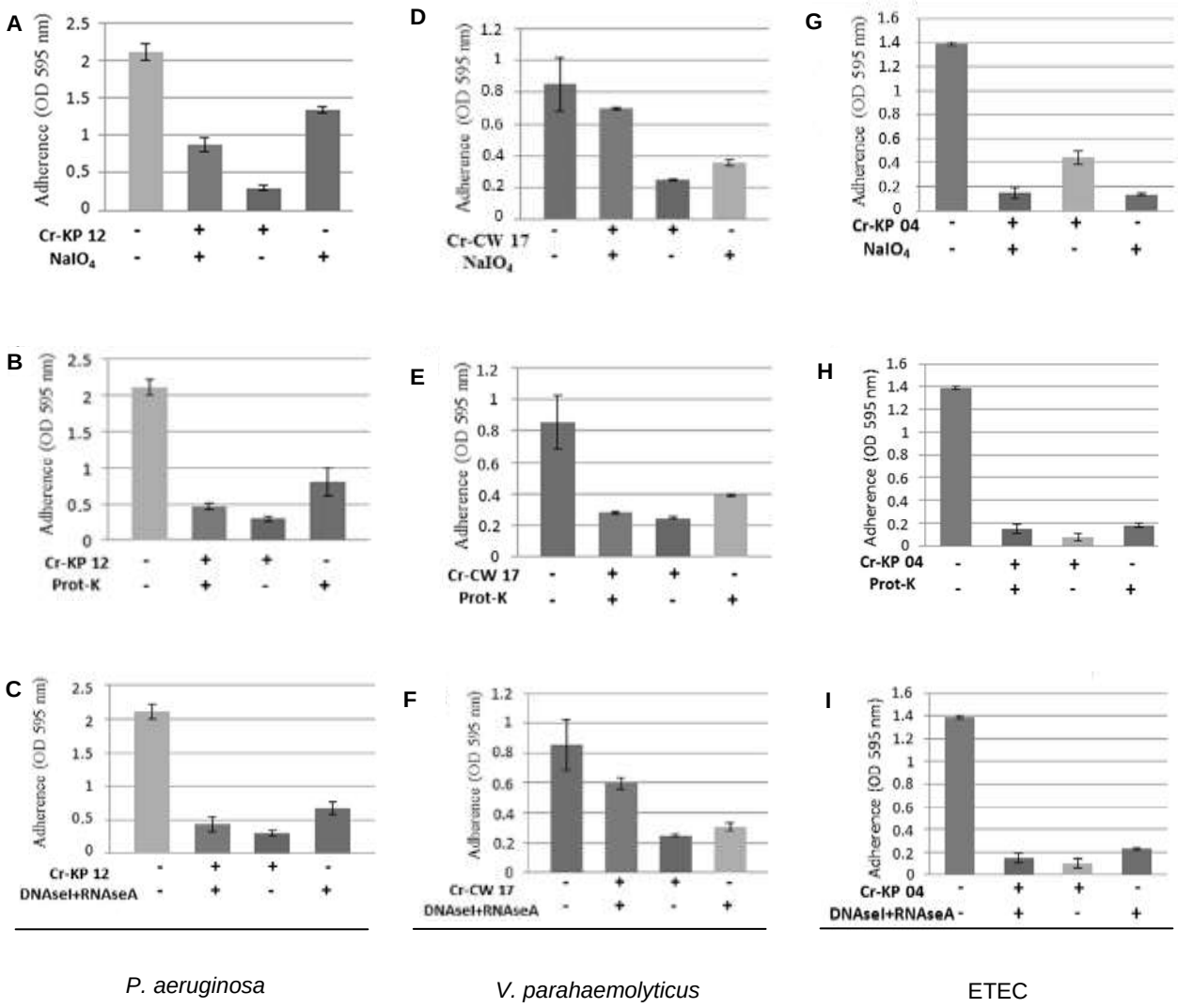

Figure 2: Effect of $NaIO_4$, proteinase K, and nuclease pre-treatment of actinomycetes crude extracts against Gram-negative pathogenic bacteria. Crude extract at 5% was respectively treated with $NaIO_4$ (20 mM), proteinase K (1 mg/mL), DNase I (100 mg/mL) + RNase A (25 mg/mL) at 37 °C for 12 h. Crude extract KP12 against *P. aeruginosa* (A-C), crude extract CW17 against *V. parahaemolyticus* (D-F), and crude extract KP04 against ETEC (G-I).

DISCUSSION

Actinomycetes bacteria produce about 70% of the antibiotics that exist. These antibiotics have many effects, including antibacterial, antifungal, antitumor, antiprotozoa, and antiviral properties. Aminoglycoside, anthracyclines, peptides, polyenes, polyketides, actinomycines, chloramphenicol, β-lactam, tetracyclines and macrolides are examples of antibiotics produced by actinomycetes. Additionally, almost the entire secondary metabolite product from actinomycetes can be applied to many biotechnology applications (Oskay *et al.*, 2004). There is much research reporting on the finding of antibiofilm bacteria from marine, soil, or arctic actinomycetes, but only one study has reported the finding of antibiofilm activity from Indonesia marine sediment (Vidyawan and Waturangi, 2013).

In a previous study, we reported that ten isolates of actinomycetes could inhibit and destroy the biofilm formation of *V. cholerae*. In this study, the same isolate extracts proved to have a broad spectrum of antibiofilm activity because all of extracts were able to inhibit and disperse the biofilm formation of Gram-positive pathogens (*S. aureus*, *S. pneumoniae*, *E. faecalis*) and Gram-negative pathogens (*V. cholerae*, *V. parahaemolyticus*, *P. aeruginosa*, ETEC) without inhibiting bacterial growth (You *et al.*, 2007). The broad spectrum of antibiofilms activity might help actinomycetes gain a competitive edge in marine sediment against other bacteria (Sayem *et al.*, 2012). Most of the isolates that showed the highest

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

inhibition are *Streptomyces* sp. These *Streptomyces* sp. isolates have shown that polysacharide and protein are their main active compounds acting to reduce the biofilm (Sayem *et al.*, 2012).

In this study, selected extracts were treated with $NaIO_4$, proteinase K, and DNAse+RNAse. $NaIO_4$ is able to hydrolyze polysaccharides by oxidizing the carbons bearing vicinal hydroxyl groups and by cleaving the C-C bonds (Jiang *et al.*, 2011). Meanwhile, treatment with proteinase K was used to degrade protein and DNase+RNase were used to degrade nucleic acid. Disrupting the multicellular structure of a bacterial biofilm was proposed as one of the most promising strategies for increasing the sensitivity of pathogens in biofilm to antibiotics and host immune systems. The activity mechanism, such as enzymes dissolving the matrix polymers of the biofilm (Nemoto *et al.*, 2000), chemical reactions blocking biofilm matrix synthesis, and analogues of microbial signaling molecules interfering with cell-to-cell communication, required for normal biofilm formation.

After characterization of all actinomycetes extracts, polysaccharides are the most likely bioactive compound for biofilm inhibition and destruction of tested pathogens. Based on the findings, active polysaccharide from actinomycetes might interfere with cell-surface influence and cell-cell interactions, which is a pre-requisite for biofilm development (O'Toole *et al.*, 2000). It has been reported in other cases that polysaccharides can produce anti-adherence effects between microorganisms and surfaces (Langille *et al.*, 2000). This anti-adherence effect might be caused by a polysaccharide that acts as surfactan molecules that modify the physical characteristics of bacterial cell surfaces. Some studies have also indicated that a polysaccharide might act as a signaling molecule that modulates gene expression of recipient bacteria (Carpenter, 1971) or acts as competitive inhibition of multivalent carbohydrate-protein interactions (Wittschier *et al.*, 2009). Thus, antibiofilm polysaccharides might block lectins or sugar binding proteins present on the surface of bacteria, or block tip adhesins of fimbriae and pili. For example, lectin-dependent adhesion of pathogenic *P. aeruginosa* to human cells is efficiently inhibited by galactomannans (Zinger and Gilboa, 2009).

Another important role in bacterial biofilm formation is cell-surface and cell-cell interaction (O'Toole *et al.*, 2000). Many bioactive compound secreted by bacteria have been reported to mediate adhesion and deadhesion between microorganisms and a surface. A receptor polysaccharide recognized by complementary protein adhesion was reported to mediate establishment of a productive cell to a surface and in cell-to-cell contacts. The ability to inhibit and destroy a cell-surface or cell-cell interaction could induce an antibiofilm effect (Jiang *et al.*, 2011).

Recent findings showed that *V. cholerae* biofilm selectively retains secreted proteins. For example, RbmA and Bap1. RbmA functions similarly to previously identified biofilm matrix proteins in that it strengthens intercellular interactions. Bap1 is jointly synthesized by biofilm-associated bacteria and is concentrated at the base of the biofilm where it reinforces the association of the biofilm with the surface and accelerates attachment of bystander bacteria not yet primed for biofilm matrix synthesis (Absalon *et al.*, 2011). *Staphylococcus aureus* also has this kind of system. Protein is one component of a *S. aureus* biofilm matrix and protease activity is required for biofilm detachment (Bolse and Horswill, 2008). Biofilm formation required protein synthesis, and it is thought that extracytoplasmic proteins as surface-exposed proteins, played a role in bacterial attachment to abiotic surfaces (O'Toole *et al.*, 2000). As such, it is possible that this extracellular enzyme produced by *Streptomyces* sp. interferes with this kind of protein that exists in the biofilm and could inhibit the formation of biofilm in *S. aureus.*

Extracellular DNA was found to be the most bioactive compound of *E. faecalis*. Extracellular DNA has been implicated as a major structural component for the initial establishment of biofilms in bacteria that specifically release DNA (Whitchurch *et al.*, 2002). Moreover, it has been recently observed that free DNA plays an important role in biofilm formation by *S. mutans*. DNA has been identified as a key structural component of the biofilm extracellular matrix and, more recently, biofilm formation was shown to involve a functional DNA-binding uptake system (Petersen and Scheie, 2005). As antibiofilm compound, extracellular DNA able to interfere formation of biofilm by binding to the adhesive structure of the planktonic cell needed for attachment to the surface (Berne *et al.*, 2011).

Although the major antibiofilm bioacive compounds came from the three components (polysaccharide, protein, or nucleic acid), some bacteria produces various kind of compound from small molecules, such as, D-amino acids, aryl rhodanines, and chelators (Chen *et al.*, 2013) to fatty acid (Rajalaksmi *et al.*, 2014) which has antibiofilm activity. One of the fatty acid crude extract which has antibiofilm activity against *Streptococcus pyogenes* came from soil *Streptomyces sps* (Rajalaksmi *et al.*, 2014). The fatty acid crude extract able to inhibit the formation of *S. pyogenes* biofilm at MIC 10 µg/mL and changes extracellular protein expression which was likely the virulence factor of the bacteria (Rajalaksmi *et al.*, 2014).

This research needs further study to characterize the structure of extracellular polysaccharide and protein from the ten isolates of actinomycetes in our study in order to obtain more information about the mechanisms of the antibiofilm effect against other pathogens. Purification of these compounds is still needed to obtain higher antibiofilm activity for further application in the medical field.

CONCLUSION

Extracts of actinomycetes from previous studies had broad spectrum antibiofilm activity. Extracts with the highest activity for antibiofilm inhibition were extracted from *Streptomyces* sp. For the characterization of bioactive compound, one isolate had a nucleic acid, one isolate had a combination of the three component

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

(polysaccharide, protein, and nucleic acid), and one isolate had a combination of polysaccharide and nucleic acid as the active compound for antibiofilm activity. The mechanisms of polysaccharides, protein and nucleic acid for inhibition of biofilm formation are by interfering and interrupting the cell-surface and cell-cell interactions, which is a pre-requisite for biofilm development. Thus, the bioactive crude extracts are very promising for application against biofilms related-infection. Further research is needed to purify the bioactive compound from the crude extracts, which has antibiofilm activity against Gram-positive and Gram-negative pathogens.

ACKNOWLEDGEMENT

This work was funded by a grant of Indonesian Federal Ministry of Education to Faculty of Biotecnology, Atma Jaya Catholic University of Indonesia.

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