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Identification of antimicrobial compounds produced by *Pseudomonas aeruginosa* NS3symbionascidian *Phallusia julinea*

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Abstract: Marine organisms have developed exquisitely complex biological mechanisms and they are known as source producing bioactive compound. The objective of this study is to test cell-free *P. aeruginosa* NS3 on pathogenic microbes and to identify antimicrobial compounds in supernatants. The method bacterial culture of *P. aeruginosa* NS3 was harvested on 72hour at the exponential growth phase and it was then centrifuged. Cell-free supernatants were tested on pathogenic microbes using disc diffusion method, the pigment components were characterized by UV-vis and weightmolecules of bioactive secondary metabolites was identified using LC-MS/MS. The result showed that cell-free supernatants of *P. Aeruginosa* NS3 could inhibit pathogenic microbes with MRSA inhibition zone of 8 ± 0.5 mm, *S. aureus* 25 ± 2.7 mm, EPEC30 ± 5.2 mm and *Candida albicans* 22 ± 3.5 mm. The minimum inhibitory concentration ranged from 31.25 to 500 ppm. The *P. aeruginosa*NS3 bacteria produced phenazine antimicrobial compounds with pyocyanin component (211m/z), 1-hydroxyphenazine (196m/z), phenazine-1-carboxamide (179m/z) and phenazine-1-carboxylic acid (155 m/z).*P. aeruginosa*NS3 bacteria producedphenazine-containing compound that has the ability to inhibit pathogenic microbes. The greatest inhibitors were EPEC, *S. aureus, C. albicans* and MRSA. It is necessary to conduct further research which uses their bioactive compounds in pharmaceuticals such as antiviral, anticancer, and anti-inflammatory.
Key words: antimicrobial activity, bioactive compounds, phenazine, free-cell supernatants,*P. aeruginosa*NS3.

Introduction

Recently, there have been many microbesresistant to antibiotics and it is necessary to find new antibiotics that can inhibit resistant bacteria and can meet the elements of producing an antibiotic. Marine microbes are a potential source of bioactive compounds that have become a concern and have been used asantimicrobials, anticancer, anti-inflammatory, antiviral, antinematoda, and antiprotozoa[1]. The bacteria may live in the water free and there are also bacteria which are associated with a variety of marine organisms such as ascidians. The mutual association of bacteria and ascidians will produce a variety of bioactive compounds such as antimicrobial compounds. Through the production of antimicrobial compounds, bacteria can adapt to the environment and defend themselves from other bacteria and their host [2,3,4].

In the previous research, *Pseudomonas aeruginosa* NS3 bacteria which had been associated with *Phallusia julinea* ascidian had been isolated. These bacteria which had a potential to produce bioactive compounds belong tothe Gamma Proteobacteria class with these characteristics: Gram-negative, rod-shaped, motile, forming smooth spherical colonies with greenish fluorescent color, wide spread in the environment. These bacteria produce pyocyanin pigment which is soluble in water, chloroform and n-butanol[5,6,7,8]. The objective of this research was to test the cell-free supernatants of *P. aeruginosa* NS3 onpathogenic microbes and to identify antimicrobial compounds in the supernatants.

Material and Methods

Growth medium

P. aeruginosa NS3 was isolated from the *Phallusia julinea* ascidian which found in Doreri Gulf, Manokwari, West Papua. The isolates were cultured in slantsea water complete (SWC)mediawith the composition of 5g/L bacto-peptone, 1 g/L yeast extract and 3 ml/L glycerol and 15 g bacto agar [9]. Bacterial colonies were incubated on media SWC slantfor 24 hours at the temperature of 30 ^{o}C andused to measure the biomass density.

Biomass density measurement

Bacterial biomass was measured based on the*P. aeruginosa* NS3 growth-curve using turbidimetric method. Oneloope of *P. aeruginosa* cultured into a 250-ml Erlenmeyer containing 150 mL SWC broth, then it was incubated in a shaker incubator at30 ^{o}C, pH 7, with the agitation of 120 rpm for 24 hours and the optical density (OD) was measured on 0,1,2,4,6,8,10,14,18,24,36,48,58,72, and 92 hour. The isolates growth was measured using a spectrophotometer (Shimadzu 1240) at a wavelength of 620 nm. The growth curve showed the relationship between the incubation time and the biomass density. Culture isolates used to test the antimicrobial was the ones which had reached the highest exponential growth phase.

P. aeruginosa NS3 antimicrobial activitytest

Culture of *P. aeruginosa* NS3 were centrifuged at a speed of 10,000 rpm, at 4 ^{o}C for 15 minutes to separate the supernatants and the cell mass. The supernatants were filtered with a 0.22 µM milipore syringe filter. The cell-free supernatant was used to test the antimicrobial on methicillin-resistant of *Staphyloccocus aureus* (MRSA), *S. aureus*, EPEC and *C. albicans* using disc diffusion method.Culture of microbes0.1 mL was spread on the SWC and Potato DextroseAgarmedia. Disc paper Ø 5 mm which had been soaked in 50 µL of bacterial culture supernatant was placed on the surface of the test medium. Cultures in a Petri dish were incubated for 24 hours at 30 °C, then, a clear zone formed was observed and its diameter was measured [10].

Determination ofminimum inhibitory concentrationof*P. aeruginosa* NS3 supernatant

The minimum inhibitory concentration (MIC) determination was done by dilution serial of *P. aeruginosa* NS3cell-free supernatant. As a result, serial concentration was obtained (2000; 1000; 500; 250; 125; 62.5; 31.5) ppm. Tube number one until seven was filled with0.1 mL pathogenic bacterial cultures. All tubes were homogenizedfor 2 minutes and incubated for 24 hours at the temperature of30 ^{o}C. Antimicrobial activity would be noticed if there was a decrease in culture optical density measured by a spectrophotometer at the wavelength of 620 nm. Antimicrobial supernatant MIC value was obtained at the highest level of dilution which could inhibit pathogenic microbes[11].

Analysis of *P. aeruginosa*NS3antimicrobial compounds

Supernatant of bacterial isolates were subsequently precipitated by the addition of chloroform (2: 1), homogenized for 1 minute and 0.1 N HCl was later added until the color changed[12]. These pigments are characterized by UV-visible spectrophotometer (UV-1600) with the absorbance of 340 nm and identified by liquid chromatography electro spray ionization-mass spectrometry analysis (LC-MS / MS) to determine the secondary metabolites molecular weight. Instrument used was triple quadrupole MS instrument (Thermo Scientific, USA). Detector used was a 3200 QTRAP mass spectrometer, Phenomenex C18 column (50 mm x 2mm) with flow rate of 0.2 ml/min, analysis time of 5 minutes, injection volume of 5 µL, column temperature

of 25 ^{o}C, gradient elution flow of 10% eluen B. Mobile phase A consisted of a mixture of distilled water and glacial acetic acid, mobile phase B: acetonitrile[13].

Results and Discussion

*P. aeruginosa*NS3 growth patterns were determined based on the biomass density with turbidimetric method and directly counting the cells under a microscope using haemocytometer. *P. aeruginosa*NS3 bacteria required nutrition containing mineral and suitable environmental conditions for optimum growth. In this study, the observations of growth curve in Figure 1 showed the exponential phase occurred on 6 to 72 hours and later experienced the decrease in number of cells or the death phase.

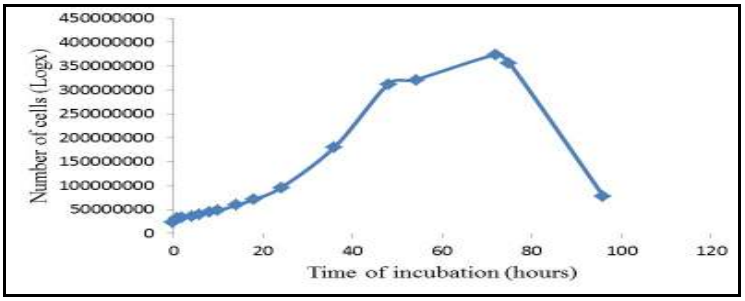

Figure 1. Growth curve of *P. aeruginosa* NS3

At the growth the exponential phase antimicrobial compound has been formed so that the biomass harvest was done at the end of the exponential phase [14]. *P. aeruginosa*NS3 bacterial cultures were harvested on 72nd hours and then separated using a centrifuge for 15 minutes at the speed of 10.000 rpm at the temperature of 4 °C. The supernatant was used to the antimicrobial activity assay.

Antimicrobial compound activity assay used the disc diffusion method. The results showed that *P. aeruginosa*NS3 supernatant could inhibit pathogenic microbes Table 1. Activity test showed different responses of pathogenic microbes towards the minimum inhibitory concentration. At the concentration of 500 ppm, the antimicrobial substance was able to inhibit the growth of Gram positive bacteria that was MRSA and *S. aureus*, whereas at the concentration of 250 ppm, it could inhibit the growth of EPEC and at 31.25 ppm, it could inhibit the growth of *C .albicans*.

Table 1. Diameters of the zones of inhibition (mm) and the MIC by cell-free supernatant *Pseudomonas aeruginosa* NS3

Microbial pathogens	Diameters fo inhibition Zone (mm)*	MIC (ppm)
MRSA	8±0.5	500
S. aureus	25±2.7	500
EPEC	30±5.2	250
C. albicans	22±3.5	31.25

*) The average value and standard deviation with triplicate measurement for each value

From MIC test results, it was found that the greater concentration usedhas greater the inhibitory activity. The higher of the concentration of an antimicrobial used, the more quickly the microbes killed[15].This is not recommended for a medical treatment because it can cause resistance. The minimum inhibitory concentration required by MRSA and *S. aureus* was higher because the bacteria have thicker cell wall compared to others.

The identification results of active compounds produced by *P. aeruginosa*NS3 bacterium showed that the bacterium producedphenazine. It is obvious that when the chloroform was added, the supernatant changed its color to bluish and when 0.1 N HCL was added, it changed to reddish. The supernatant was analyzed using UV-visible spectrophotometer to confirm the presence of the phenazine by generating absorption at the wavelength of 279, -343, -369, -372 nm Figure 2.

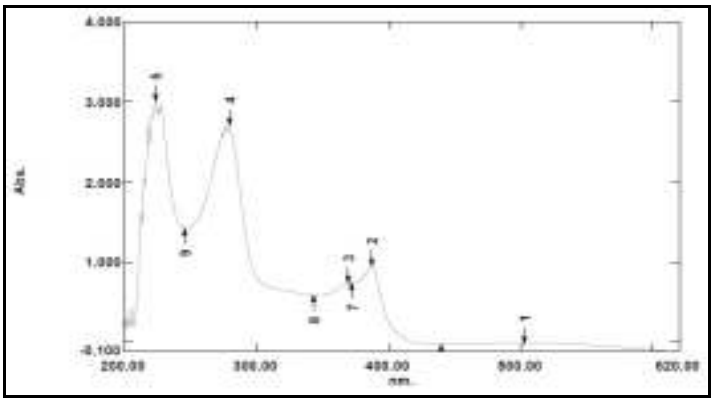

Figure 2. UV absorption spectra of *P. aeruginosa* NS3 showing λ_{max} 340nm

The supernatant was analyzed by LC-MS / MS to determine the molecular weight of the components Figure 3. Phenazine components produced by*P. aeruginosa*NS3 were pyocyanin(211m/z), 1-hydroxyphenazine (196m/z), phenazine-1-carboxamide (179m/z) and phenazine-1-carboxylic acid (155m/z).

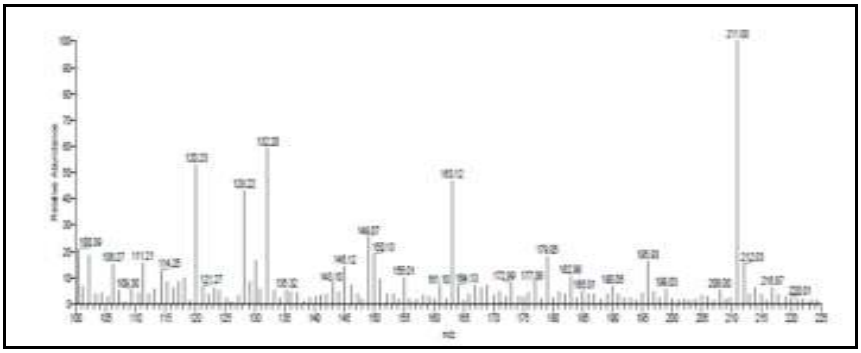

Figure 3. LC-MS/MS analyses of phenazine compounds produced by *P. aeruginosa* NS3

The phenazine contained heterocyclic nitrogen and possessed the activity of antimicrobial, antitumor, and antiparasitic[16,17]. Some studies suggest that phenazine efficiently inhibits bacteria and fungi[8,18,19]. The phenazine wasproductedduring the stationary phase [5]. Active mechanism of the phenazine compound was by damaging the cell membrane, inhibiting the synthesis of nucleic acids and proteins [7].

Conclusion

*P. aeruginosa*NS3bacteria which were isolated from *P. julinea* ascidian found in Doreri Gulf, Manokwari, West Papua had antimicrobial activity. The antimicrobial substance produced by *P. aeruginosa*NS3could inhibit pathogenic microbes MRSA, *S. aureus*, EPEC and *C. albicans*.

The antimicrobial compound from*P. aeruginosa*NS3 bacteria was phenazine consist of pyocyanin, 1-hydroxyphenazine, phenazine-1-carboxamide and phenazine-1-carboxylic acid.

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