

Antibacterial Activity of Carotenoid from Bacterial Symbiont of the Soft Coral *Sinularia* sp. against MDR and MRSA Bacteria

Yuvianti Dwi Franyoto¹, Ahmad Fuad Masduqi¹, Nasa Ayuning Swastiwi¹,
Muhamad Ramanda Sholihulhadi¹, Handung Nuryadi², Lia Kusmita^{1,*}

¹STIFAR Yayasan Pharmasi Semarang Letjend Sarwo Edhie Wibobo Km 1, Semarang, Indonesia

² Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Okinawa, 903-0213, Japan

*Corresponding Author: lia_kusmita@yahoo.com

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ABSTRACT

This study aimed to isolate and identify the soft coral symbiotic bacteria *Sinularia* sp. which has activity against pathogenic bacteria MDR (*Escherichia coli* and *Staphylococcus aureus*) and MRSA (methicillin-resistant *Staphylococcus aureus*). Symbiotic bacteria isolated from the soft coral *Sinularia* sp. were collected from the waters of Panjang Island, Jepara, Central Java, Indonesia for antimicrobial activity. Carotenoid-containing bacteria were tested for antibacterial activity against various pathogenic bacteria, including MDR *Escherichia coli*, MDR *Staphylococcus aureus*, and MRSA and identifying antibiotic producer which is potential. The carotenoid extracts obtained were identified by UV-Vis spectrophotometer. The results showed that the P.Si 1 isolate was symbiotic bacterial from *Sinularia* sp. containing carotenoids. The molecular identification based on 16S rDNA showed that the most closely related species with P.Si 1 was *Acinetobacter vivianii* with only 80% of sequence identity. The results of the screening test showed that the average clear zone diameter for MDR *E. coli* antibacterial test at 2% concentration was 1.243cm, at 3% was 1.476cm, and at 4% it was 1.765cm, with a positive control of 2.325cm. The results of the antibacterial test against MDR *S.aureus* recorded average diameters at concentrations of 2% (1.534cm), 3% (1.678cm), 4% (1.885cm), with a positive control of 2.543cm. In addition, the antibacterial test against MRSA showed average diameters at concentrations of 2% (1.757cm), 3% (1.944cm), and 4% (2.247cm), with a positive control of 2.471cm. Remarkably, carotenoids in *A. vivianii* have antibacterial activity.

INTRODUCTION

Diseases caused by pathogenic bacterial infections have become a major problem in the field of health. The improper and uncontrolled use of antibiotics against pathogenic bacteria has resulted in antimicrobial resistance, which has become a major health problem worldwide (Goldmann *et al.*, 1997; Radjasa *et al.*, 2007). Antibiotic-producing microbes can be either fungi or bacteria, which are usually symbiotic in other organisms such as coral reefs (Faulkner *et al.*, 2000).

Harper *et al.* (2001) stated that, chemical compounds produced from coral reefs are useful for preventing and maintaining predatory attacks, media competition, preventing bacterial infections and ultraviolet light stings, and assisting the reproductive process. The continuous use of bioactive compounds in soft corals can threaten their sustainability in the waters because the growth of these biota is relatively slow. According to **Arafat (2009)**, the growth rate of *Sinularia* species is around 0.07- 0.24cm per month, with width ranging from 0.08- 0.28cm per month. Therefore, various efforts have been made to maintain these resources in nature, including studies of microorganisms that are symbiotic with soft corals.

The bacterial symbiosis with marine invertebrates can synthesize active compounds identical to hosts (**Perez-Matos *et al.*, 2007**). Symbiotic bacteria are able to rapidly produce the content of active compounds so that they can be produced more easily, quickly and much on the scale of biotechnology than soft coral culture itself (**Thiel, 2006**). Soft coral symbiotic bacteria can synthesize similar secondary metabolites with their hosts (**Burgess *et al.*, 2003**). This allows bacterial symbionts to produce antibacterial compounds that can be used as therapeutic agents for some diseases caused by multidrug resistant (MDR) bacteria.

Some symbiotic bacteria are useful as a source of producing secondary metabolites such as natural pigments (**Radjasa *et al.*, 2003; Kusmita *et al.*, 2021**). Several heterotrophic bacteria that synthesize carotenoids have also been isolated from the coasts and oceans (**Du *et al.*, 2006; Satsnes *et al.*, 2010**). Based on the data above, further research on carotenoid pigment from *Sinularia* sp symbiont bacteria could be potential as an antibacterial agent against MDR (*Escherichia coli* and *Staphylococcus aureus*) and methicillin-resistant *Staphylococcus aureus* (MRSA).

MATERIALS AND METHODS

Collection and preparation sample

Soft coral samples of *Sinularia* sp. were collected from the Panjang islands, Jepara, North Java Sea, Indonesia. Soft coral samples were gathered at approximately 2 meters depth. Small pieces of the tissue of *Sinularia* sp. were cut and placed into a plastic bag. On the surface, collected samples were rinsed with sterilized sea water to remove sands and sediments. Subsequently, tissue samples of *Sinularia* sp. were brought to the laboratory for symbiotic bacterial isolation.

Isolation of symbiont bacteria

Bacterial isolation was done using the distribution method of **Radjasa *et al.* (2007)**. Tissues of *Sinularia* sp. were cut with a sterile knife, and then crushed with sterile mortar and stamper. Approximately, 1g of samples was put into a test tube containing 9 mL of sterilized seawater and mixed well. From the treatment obtained, 1mL of 10^{-1} dilution was transferred into a test tube containing 9 mL of sterile sea water to obtain 10^{-2} dilution. This step was repeated to obtain serial dilutions of 10^{-3} , 10^{-4} and 10^{-5} . Furthermore, 35 μ L of solution from each dilution was inoculated into a petri dish containing solid Zobell media. Inoculated samples were incubated at 37°C for 2x24 hours. Bacterial colonies were observed based on the color, shape, edge and surface. Then, colored bacteria were purified using streak method.

Molecular identification

Molecular identification was carried out using polymerase chain reaction (PCR) 16S rDNA. The universal primers for bacteria, 27F (5'-AGAGTTTGAT CMTGGCTCAG-3') and 1492R (5'TACGGYTACCTTGTTACGACTT-3') were used for PCR amplification. The PCR cycles were at 96°C for 2 minutes, followed by 35 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. PCR products were visualized using 1% agarose gel to observe quality of the PCR products. Good quality of PCR products were sent to (company name) to obtain nucleotide sequences. Basic local alignment search tool (BLAST) was used to detect closely related species from database of GenBank with our isolated bacterial strains (Altschul *et al.*, 1997; Radjasa *et al.*, 2013).

Extraction of carotenoid

Carotenoid pigments were extracted using methanol according to Kusmita *et al.*, 2017. Cell pellets were macerated in methanol until cell pellets become colorless. Pigment extraction was performed in dark condition. Subsequently, centrifugation at 5,000 rpm for 5 minutes was performed to separate pigment solution and cell pellets. Extracted pigments were then dried using rotary evaporator and nitrogen gas.

Carotenoid Pigment Identification

Initial identification of pigments from symbiotic bacteria was analyzed using visible spectrophotometer at a wavelength of 300-800 nm. Then, the spectra pattern was observed in the carotenoid area at a wavelength of 300-600 nm (Gross, 1991).

Antibacterial activity test

Crude pigment extracts were dissolved in dimethyl sulfoxide (DMSO) solvent to make extract solution with concentrations 2%, 3% and 4% (m/v) for antibacterial activity test. The extract solutions with various concentrations were tested against MDR (*Escherichia coli* and *Staphylococcus aureus*) and MRSA using the diffusion agar method with perforation techniques. Amoxicillin trihydrate 0.05% b/v was used as a comparison control. Media containing bacterial suspension and extraction of symbiotic bacterial carotenoid pigments were incubated at 37 °C for 1 x 24 hours. The antimicrobial activity was determined by observing the inhibitory zones. The inhibitory zone around the paper disks indicated that carotenoid pigment extracts of symbiont bacteria isolates were able to inhibit the growth of tested bacteria (Radjasa *et al.*, 2007; Murti *et al.*, 2012).

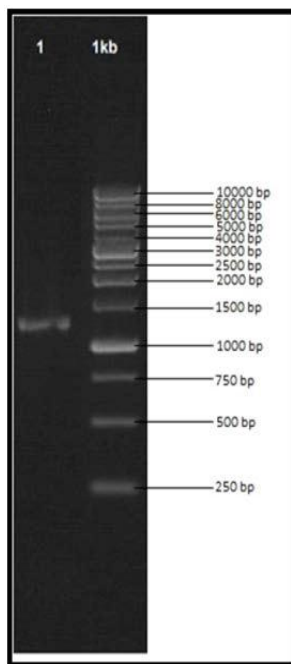
RESULTS

A total four bacterial strains were successfully isolated from *Sinularia* sp. Those bacterial colonies have presented different morphologies (Table 1).

Carotenoid-containing bacteria were then purified to obtain a pure single colony (Fig. 1). Molecular analyses based on 16S rDNA were carried out to identify selected bacterial isolate. This identification aims to determine the identity of symbiont bacteria. In gel electrophoresis visualization, there is a DNA band which indicates that the amplification process was successfully carried out (Fig. 2).

Table 1. Results of Isolation of Symbiont Bacteria *Sinularia* sp.

No	Code	Color	Shape	Surface	Edge
1.	P.Si 1	Yellow	Round	Curved	Complete
2.	P.Si 2	White	Round	Curved	Complete
3.	P.Si 3	White	Irregular	Curved	Choppy
4.	P.Si 4	White bone	Irregular	Curved	Choppy

**Fig 1.** Purified bacterium of P.Si. 1 isolate**Fig. 2.** Visualization of Amplification Results

The next analysis uses BLAST (Basic Local Alignment Search) through the site <http://www.ncbi.nlm.nih.gov/>. DNA sequencing with BLAST was done to compare 16S rDNA sequences from P.Si. 1 bacterial isolate with the world DNA worldwide DNA

database. The worldwide DNA database is always updated on a daily basis and cross checks are carried out among three world DNA databases, namely Gen Bank, DNA database of Japan (DDJB), and European Molecular Biology Laboratory (EMBL), so that the sequence status will not overlap and is always up to date. Based on the results of BLAST that has been done, P.Si 1 bacteria is identical to the bacterium species *Acinetobacter vivianii* with homology of 80% (Table 2).

Table 2. Results of BLAST Homology Search on P.Si 1 Bacteria

Sample Code	Length (bp)	Closest relative	Accession Number	Homology (%)
P.Si 1	1270	<i>Acinetobacter vivianii</i>	NR_148847.1	80

The BLAST analysis results were then confirmed by using a phylogenetic tree from the P.Si 1 strain using the Molecular Evolutionary Genetics Analysis (MEGA) software version 6.06 (Fig. 3).

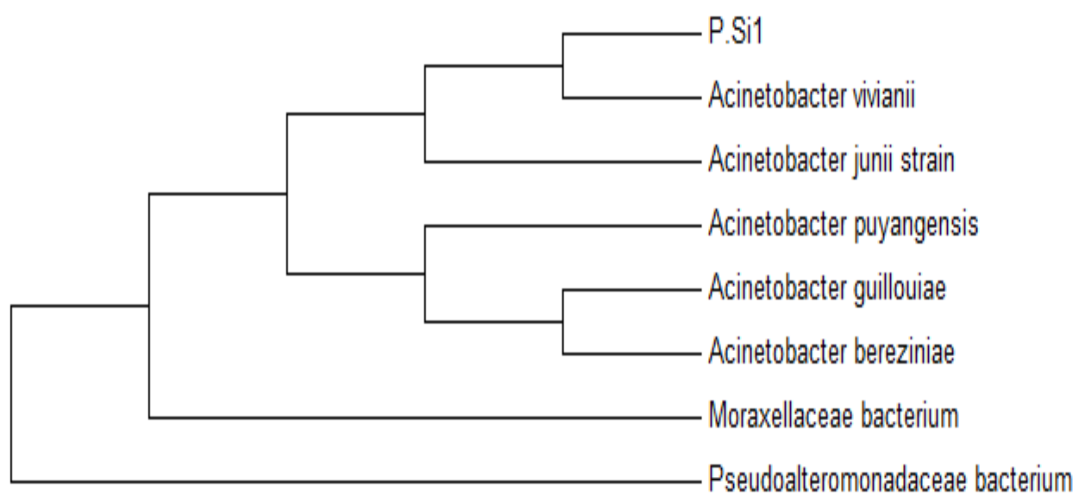


Fig. 3. Phylogenetic Tree of Bacteria P.Si 1

The results of the measurement of the pigment patterns show that three peaks were detected at the range 300-600 nm (Fig. 4).

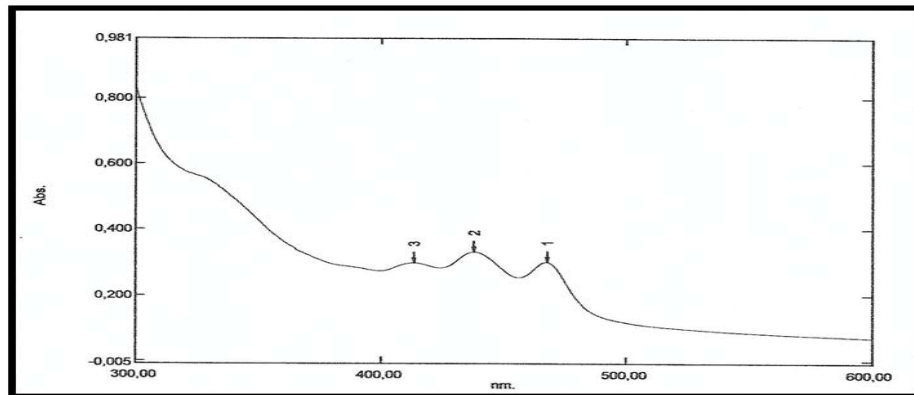


Fig. 4. Carotenoid Pigment Spectra Pattern of P.Si 1

The results of antibacterial activity testing showed that higher concentration of carotenoid pigment extract had strong antibacterial activity. It is indicated by larger inhibitory zone formed in high concentration of tested extract (**Table 3**).

Table 3. Test of carotenoid antibacterial activity of P.Si 1 symbiont bacteria

Concentration (%) (m/v)	Diameter of the Inhibitory Zone (cm)		
	MDR <i>e.coli</i>	MDR <i>s. aureus</i>	MRSA
2	1.243±0.12	1.534±0.25	1.757±0.30
3	1.476±0.24	1.678±0.31	1.944±0.27
4	1.765±0.22	1.885±0.28	2.247±0.25
Positive control	2.325±0.17	2.543±0.26	2.471±0.22
Negative control	0,000	0,000	0,000

DISCUSSION

From four isolated bacteria, only one bacterium has a yellow color with the code P.Si 1. Based on its coloration, this isolate is suspected to contain carotenoid pigments. Carotenoids are natural yellow, orange and red pigments that are widespread in plants, algae, fungi, yeast and bacteria (**Heriyanto and Limantara, 2009**).

Based on the results of the amplification, the base length of the P.Si 1 bacterial isolate was around 1500 bp. The size of the 1500-1600 bp size of 16S rDNA sequence for the bacteria (**Sabdono, 2006**). According to **Hagström (2000)**, isolates with 16S rDNA sequence identity >97% can represent the same species, and sequence identity between 93–97% can be identified as same genus but differed at the species level. While the sequence identity <93% represents the new genus. The Sequence sequences Sequence of bacterium P.Si 1 shows the existence of a new genus. The results of tracing homology of bacterial P.Si 1 using BLAST can be seen in Table 2.

Phylogenetic trees are useful to show the relationships of each species based on molecular characteristics between species and between strains within the same species (**Felix et al., 2011**). Bacteria *Acinetobacter vivianii* is a gram negative bacterium that belongs to the Kingdom Bacteria, Protobacteria Phylum, Gammaproteobacteria Classes, Alteromonadales Order, Moraxellaceae Family, Genus *Acinetobacter*, and species *Acinetobacter vivianii*. This bacterial species is an aerobic bacterium that can grow in

mineral media with acetate as a carbon source and ammonia as a nitrogen source. On media of Tryptic Soy Agar, this bacterium has a diameter of 1.5-2.0 mm, is gray / white, round in shape, the complete, the surface is convex and smooth. The optimum temperature of bacteria growth of *A. vivianii* is 15°C - 37°C (**Nemec et al., 2016**). Bacteria *A. vivianii* can be found in marine waters and are attached to rock surfaces, algae, coral reefs, marine animals, marine sediments, and also were found in coastal waters (**Gaurthier et al., 1976**).

According to (**Kusmita et al., 2017**), methanol is effective to extract pigments from bacterial cells, including carotenoid pigments. Identification of carotenoid pigment content was carried out using a UV visible spectrophotometer with a wavelength range of 300-800 nm. According to **Gross (1991)**, carotenoid pigments have absorption around 300-600 nm, namely in the red area. Based on these results, it can be concluded that bacterium P.Si. 1 can produce carotenoid pigments. Carotenoids from symbiont bacteria have antioxidant and antiultraviolet activity (**Kusmita et al., 2021**), as well as antibacterial activity (**Kusmita et al., 2021**).

The ability of the sample to inhibit the growth of Methicillin Resistant *Staphylococcus aureus* (MRSA) bacteria is due to the presence of carotenoids which are groups of terpenoids (**Del Campo et al., 2007**). Many carotenoid pigments are reported to have potential biological activities, such as antimicrobial and antioxidant (**Manimala and Murugesan, 2014**). The mechanism of action of carotenoid compounds as antibacterial substances involves damage to the membrane by lipophilic compounds and accumulation of lysozyme enzymes that digest the bacterial cell wall (**Sahnnon and Abu Gannam, 2016**). Carotenoids can react with porins (transmembrane proteins) on the outer membrane of bacterial cell walls, forming strong polymer bonds and damaging porins. Porins are the entrance and exit of the compound from and into the cell. Damage to the porin will reduce the permeability of the bacterial cell wall and will result in nutrient-deficient bacterial cells, so that bacterial growth becomes inhibited or dead (**Cowan, 1999**).

CONCLUSION

The bacterial symbiont of *Sinularia* sp. may have potency as a sustainable carotenoid bioresource. Carotenoid from P.Si 1 bacterium have antibacterial potential against MDR and MRSA strain. The closely related species with Isolate P.Si1 was *Acinetobacter vivianii* with sequence identity only 80%. Hence, this isolate can be used and developed as an antibacterial alternative.

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