Characterization of Carotenoids from Bacterial Symbiont Virgibacillus salarius Strain 19.PP.Sc1.6 from Panjang Island, Jepara, North Java Sea Indonesia

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Research Article

Characterization of Carotenoids from Bacterial Symbiont Virgibacillus salarius Strain 19.PP.Sc1.6 from Panjang Island, Jepara, North Java Sea Indonesia

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ABSTRACT

Carotenoids are secondary metabolites commonly found in organisms on land and at sea. One of the marine organisms producing carotenoid compounds is soft corals, a slow growth and delicate group that its exploiting can damage the balance of the reef ecosystem. The use of symbiotic bacteria of soft corals is an alternative that can be developed in a sustainable way. The purpose of this research is to find out the characterization of symbiotic carotenoid-producing bacterium Virgibacillus salarius strain 19.PP.Sc1.6 of softcoral Sinularia sp. Samples of softcoral were obtained from Panjang Island, Jepara, North Java Sea, Indonesia. Carotenoid identification method was carried out by using a spectrophotometer UV-Vis, TLC, and HPLC. Antioxidant activity test used the DPPH method. Sunscreen activity was performed by the Mansyur method using a UV spectrophotometer-Vis. The results revealed that bacterial symbiont V. salarius strain 19.PP.Sc 1.6 contains carotenoids, one of which was nostoxantin. Carotenoid extract had antioxidant activity with value IC_{50} of 438 ppm. Carotenoid extract at a concentration of 438 ppm had an SPF value of 4. 97, and was considered within the medium protection category, with Te value of 29.91% and% Tp of 21.34% in the UVA sunblock category, respectively.

Keywords: carotenoids, symbiotic bacteria, antioxidants, sunscreen

INTRODUCTION

Carotenoids are natural pigments that are yellow, orange to red [1,2]. The compound is a long chain isoprene that has a conjugated double bond [3]. The conjugated double bond will reduce the oxygen singlet and deactivate other free radicals. The function of carotenoids as deactivating free radicals occurs through the electron transfer process [3,4,5]. Carotenoid pigments can be found in terrestrial and marine organisms. Carotenoids from marine organisms have distinctive characteristics, the source can be found in seaweed, microalgae, marine animals, hard corals and soft corals.

Soft coral is part of a coral reef that has been proven to produce many active compounds that can be obtained by extracting the soft coral. The problem that arises is the slow growth of soft corals, and if explored in unsustainable way, it will be very detrimental to the balance of the marine ecosystem. A strategy to overcome this problem is to utilize microorganisms associated with soft corals. The association of microorganisms with their hosts can produce the

striking metabolite compounds [6,7], including the carotenoid pigments.
Several studies showed that of bacterial symbiont of marine organisms produce carotenoid pigments [8.9]. In addition, [9,10] also reported that the bacterial symbiont associated with Sarchophyton have antioxidant and anti ultraviolet activity.
The antioxidant properties of carotenoids are act as singlet oxygen extinguishers and interact with free radicals [11]. Carotenoids can extinguish oxygen singlets that are formed due to the presence of sensitizers from other molecules such as: chlorophyll, porpirin and ribloflavin commonly found in biological systems. If the singlet oxygen is not deactivated by carotenoids, it will attack cells causing cell abnormalities, DNA damage and lipid peroxidation. In the process the carotenoids will return to the ground state by releasing excess energy in the form of heat or transferring energy to a stable triplet oxygen energy level [12,13,14]. Carotenoids can also react with free radicals through the charge or electron transfer process. In this reaction the charge or electrons in free radicals do not disappear, so that in a complete reaction one or more molecules remain in a radical state. All types of carotenoids are effective in eliminating oxygen singlets including carotenoids and carotene and xanthophyll groups [13]. Carotenoid radicals formed in the reaction are relatively stable and do not have enough energy to be able to react with other molecules to form new radicals [15].
Based on these functions and benefits, carotenoids from soft coral bacterial symbiont need to be developed. This study aims to develop carotenoid pigments derived from soft coral symbiotic bacteria that are fast growing and are expected not to damage the ecosystem in the sea.

MATERIALS AND METHODS

Sampling

Samples of soft coral Sinularia sp. were taken from the Panjang Island by scuba diving from a depth of approximately 2 m (**Figure 1**). After the sample taken, the colony of Sinularia sp. Was put in a plastic container containing sterile sea water and was then temporarily stored in a coolbox [9,16]. Documentation of samples of Sinularia sp. was done in situ shooting by a Canon S50 underwater camera and in the surface as soon as the sample taken from the sea.

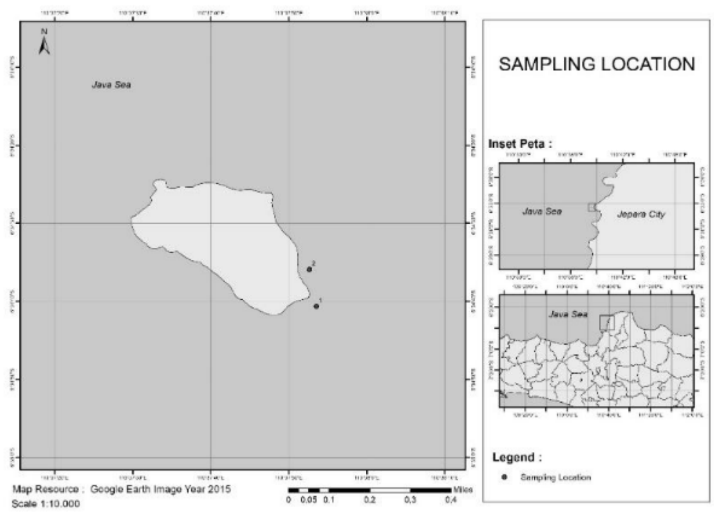


Fig.1:Sampling location of Sinularia sp. from Panjang Island

Isolation of bacterial symbiont of soft corals

Isolation of bacterial symbiont was carried out by the distribution method. Samples of softcorals were rinsed using sterile seawaters, and were cut into pieces. A series of dilutions were carried out on the sample to get a sample dilution of 10-1; 10-2; 10^{-3}; 10^{-4}; and 10^{-5}.
From each of these dilution series, 1 ml of sample was then taken and put in a sterile petri dish poured with agar media of Zobell 2216E. The petri dish were then incubated at 30°C for 1-2 days. Coloured bacterial colonies that grow on the surface of the agar media were separated by a streak method to obtain pure bacterial strains [6,7,17].

Pigment Extraction of Symbionic Bacteria
A mass of bacteria containing carotenoids was collected, extracted with methanol as a solvent using centrifuge, after the pigment is removed from the microorganism and then centrifuged again. A filtrate containing carotenoids and pellets in the form of a bacterial mass was obtained. The filtrate was then filtered with filter paper and evaporated and dried using N2 gas [9,16].

Phytochemical Screening
Carotenoid extract was evaluated by phytocemical quantitative reactions for secondary metabolite profiles. The screening was performed for phenol, flavanoids, alkaloids, saponins, terpenoids, and, tannins. The color intensity or the precipite formation was used as analytical responses to these tests [18,19,20].

Identification of Pigments from Bacterial Symbiont
Initial identification of pigment from symbionic microorganisms was carried out using a variant CARY 50 multiple beam spectrophotometer at a wavelength of 300 - 600 nm [7,921]. Furthermore, it was then identified by thin layer chromathography (TLC) using silica gel GF254 as the stationary phase, and acetone: hexane (8: 2 v / v) as the mobile phase. Finally it was characterized by using High Performance Liquid Chromathography (HPLC) Shimadzu Separative-UFLC, Kyoto, Japan using a Symmetry C8 column (150 x 4.6 mm, 3.5 µm particle size, 100 Å pore size) (wáter, Milford, MA, USA) with two eluents as mobile pase. Eluent A composed of 50% methanol and 25% acetonitrile, as well as 25% pyridine solution (0,25 M, pH 5) (v/v) and eluent B composed of 20% methanol, 60% acetonitrile, and 20% aceton. The method used refers to the published method by [22].

Antioxidant Activity
The extract was dissolved in methanol and various concentration series were made. The blank was a methanol solution, while the sample solution consisted of 4 ml DPPH plus 1 ml extract. Both the blank and the sample were incubated for 30 minutes in a dark room, then their absorbances were measured at a wavelength of 517 nm using a single UV-visible spectrophotometer Shimadzu 1240 [23].

$$\% \text{ Inhibitory} = \frac{[DPPH]_0 - [DPPH]_s}{[DPPH]_0} X100\%$$

$[DPPH]_0$ = Initial DPPH concentration
$[DPPH]_s$ = Final DPPH concentration remaining

Determination of Sun Protection Factor (SPF)
Determination of the effectiveness of protection against UV-rays was done in vitro with a UV-Vis spectrophotometer, at a wavelength of 290-320 nm. The average absorption was set at intervals of 5 nm. The carotenoid extracts used was the concentration of IC_{50} by antioxidant activity. The absorbance results were recorded then the SPF value was calculated using the Mansyur equation [24,25,26]:

$$SPF = CV \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times abs(\lambda)$$

Which are:
CF = correction factor
EE = Spectrum of erythema effect
I = spectrum of sun's intensity
Abs = Absorbance of sample

Prediction of SPF value in Table 1.

Table 1. Prediction of SPF value

SPF	Protection category
2 - 4	Minimum
4 - 6	Moderate
6 – 8	Extra
8 - 15	Maximum
≥ 15	Ultra

Determination of% transmission of erythema and% transmission of pigmentation
Absorption of the carotenoid extract was measured with a UV-Vis spectrophotometer at wavelengths that could cause erythema of 292 nm - 372 nm, every 5 nm [27]
Based on the absorption value (A) obtained, the transmission (T) was calculated by the formula:
A = - log T

Transmission of erythema (Te) calculated by formula:
$Te = T \times Fe$
Where Fe is the erythema flux value at wavelength 290-320 nm and Fp is the value of pigmentation flux at wavelength 320-375 nm [28]. The amount of erythema flux that is passed on by sunscreen (Ee) is calculated by the formula:

$$\% \text{ transmission of erythema (Te)} = \frac{Ee}{\sum Fe} = \frac{\sum (T \times Fe)}{\sum Fe}$$

$$\% \text{ transmision of pigmentation (Tp)} = \frac{Ep}{\sum Fp} = \frac{\sum (T \times Fp)}{\sum Fp}$$

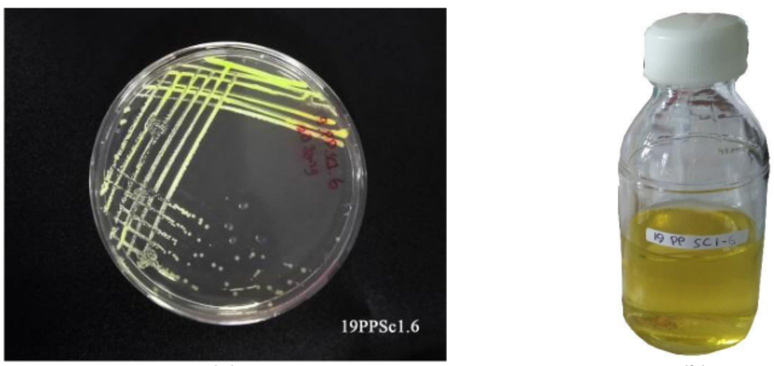
%Te and Tp can determine the sunscreen category in **Table 2**.

Table 2. The category of sunscreen activity

Category	Transmitted UV light range (%)	
	% erythema	% pigmentation
Sunblock	<1	3-40
Extra protection	1-6	42-86
Suntan	6-12	45-86
Tanning	10-18	45-86

RESULTS AND DISCUSSION

V. salarius strain 19.PP.Sc1.6 is an sample of bacterial symbiont associated with a softcoral Sinularia sp. [29]. The bacterium is yellow with a size of 1.0 mm, round shape, entire margin, and raised elevation (Figure 2). Initial screening for carotenoid pigments can be seen from the color of the bacterium. Carotenoids are pigments that are yellow to orange [21]. Extract of the bacterium V. salarius strain 19.PP.Sc1.6 is shown in figure 2.



(a) (b)

Figure 2. (a) Isolate of bacterium and (b) extract V. salarius strain 19.PP.Sc1.6

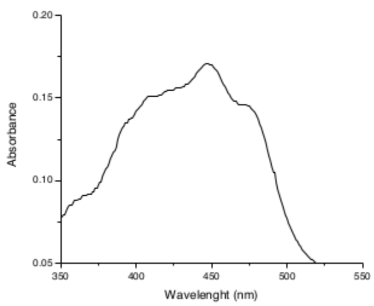
To ensure the content of secondary metabolites contained in the extract phytochemical screening was performed. The results of phytochemical screening are shown in Table 3.

Table 3. Screening results of phytochemical extracts of V.salarius strain 19.PP.Sc1.6

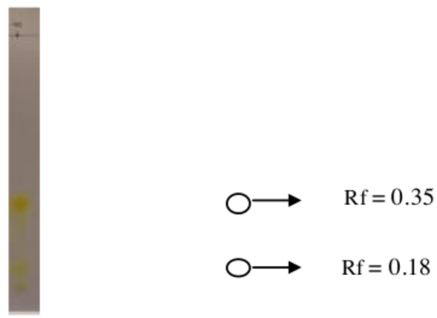
Test	Result	References	Conclusion
Phenol	A yellow solution is formed	A blue solution is formed	(-) negative
Flavonoids	A yellow solution is formed	A yellow to red solution is formed overlaying amyl alcohol	(-) negative
Alkaloids	No precipitate	Mayer reagents form white deposits, and orange sediment drag	(-) negative
Saponins	Unstable foam	Formed a stable foam that lasts for 10 minutes	(-) negative
Terpenoids	A red solution is formed	A red solution is formed	(+) positive
Tannins	No white deposits	White precipitate	(-) negative

Based on the phytochemical screening above, the extract of the bacterium V. salarius strain 19.PP.Sc1.6 positively contains terpenoids. Terpenoids are groups that have the basic structure of isoprene [30]. The most important terpenoid group is carotenoids with the molecular formula $C_{40}H_{64}$ formed by tetraterpenoids having 8 isoprene units [31,32]. Carotenoids have a straight chain structure formed by condensation of isoprene molecules [33]. Straight chain structures that have conjugated bonds can be identified using a spectrophotometer. The spectrum pattern of bacterial extracts of V. salarius strain 19.PP.Sc1.6 is shown in Figure 3a.

The results of spectrum measurements with a UV-Vis spectrophotometer showed that bacterial extracts have absorption at wavelengths of 300-500 nm and have 3 peaks (Figure 3). The spectrum shows that the compound contained in the V. salarius 19.PP.Sc1.6 bacterial symbiont is a carotenoid pigment. Based on [1], carotenoid pigments have absorption around 300-600 nm, which is in the red area. Most carotenoids absorb maximally at three wavelengths, resulting in three-peak spectra [11]. Further identification is done by TLC. The TLC results showed that there were 2 spot stains that produced yellow (Figure 3b).



(a)



(b)

Fig.3: The spectrum pattern of 19.PP.Sc1.6 bacterial extract in methanol (a) and TLC chromatogram using silica gel GF254 as the stationary phase, and acetone: hexane (8: 2 v / v) as the mobile phase (b)

The identification of carotenoid extracts using High Performance Liquid Chromatography (HPLC) Photo Diode Array (PDA). The use of HPLC with PDA detectors has the advantage of a one-time analysis that is able to identify various wavelengths from 190 - 800 nm. In addition, without using standards, the compounds could be identified from the resulting spectrum patterns.

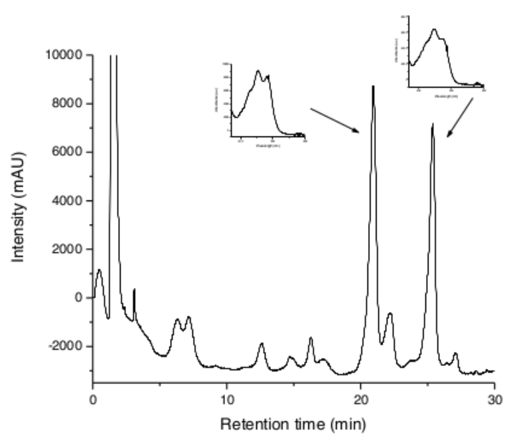


Fig.4: HPLC profile of the extracts of 19.PP.Sc1.6. The detection was carried out at 450 nm with flow rate of 1 mL per min^{-1}.

Table 4. Carotenoid identification of bacterial symbiont of 19.PP.Sc1.6

Sample	tR	Component	λmaks			Reference
19PPSc16	20.95	Nostoxanthin	(428)	455	487	Setiyono (2019)
	25.37	Unidentified	(424)	448	473	

Carotenoids found in the bacterial symbiont 19.PP.Sc1.6 consisted of 2 types. Based on [34] using the same HPLC system (column, mobile phase, elution system, detection, and flow rate) the carotenoids that appear at 20.95 minutes have the same maximum wavelength identified as nostoxanthin (p, p-carotene-2,3,2 ', 3'-tetrol) that belongs to the xanthophyll group because it has oxygen atoms [11,21]. Carotenoids of xanthophyll groups are used by photosynthetic organisms and essential for the defense of the organism [35]. The peak that appears at minute 25.37 is the carotenoid pigment [1,11], but the type of carotenoid has not been identified yet. [36] successfully isolated bacterium V. salarius TG that produced δ-carotene from the nudibranch invertebrate Jorunna funebris. The V. salarius strain CBSCP 1.1 associated with a softcoral Sarcophyton sp. from Karimunjawa Island also produced β-carotene [9].

Carotenoid bioactivity of the bacterium V. salarius strain 19.PP.Sc1.6 was tested for antioxidant activity and the sunscreen activity. The results of the antioxidant activity test using DPPH method obtained IC_{50} was 438 ppm and for comparison to the β-carotene marker was 514 ppm (Figure 5.).

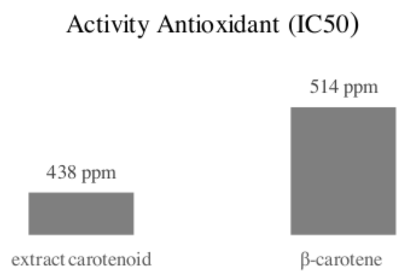


Fig.5:Antioxidant activity from extract carotenoid of baterium V. salarius 19.PP.Sc1.6 and β-carotene marker

The smaller the IC_{50}, the greater its antioxidant activity. The antioxidant activity of the carotenoid extract of the V. salarius strain 19.PP.Sc1.6 was1.2 times greater than the β-carotene marker. [9] examined the antioxidant activity of carotenoid extracts from soft coral symbionts originating from Karimunjawa showed that carotenoid extracts derived from V. salarius bacteria had the highest activity compared to other types of bacteria.

The IC_{50} concentration produced was then tested using a UV-Vis spectrophotometer to obtain the SPF value,% erythema, and% pigmentation. The test results are shown in the following Table 5.

Table 5. Assessment categories of sunscreen extracts of the V. salarius strain 19.PP.Sc1.6

	Value	Category
SPF	4.97	Medium Protection
% Te	28.91	-
% Tp	21.34	UVA Sunblock

Based on the above results V. salarius strain 19.PP.Sc1.6 carotenoid extract at a concentration of 438 ppm has an SPF value of the moderate protection category. For the ability of sunscreen seen from the value of% Te and% Tp included in the category of UVA sunblock. Sunblock is a preparation that can absorb almost all UV-B and UV-A rays if it has% Te <1% and% Tp 3-40% [37]. Include UVA category if %Te was not in category range and %Tp was in the category range [27].

CONCLUSION

The current study shows that bacterial symbiont of of softcoral Sinularia sp. from North Java sea is potential for medicinal property. Bacterial symbiont V. salarius strain 19.PP.Sc1.6 is confirmed as carotenoid-producing bacterium, especially the nostoxanthin pigment. The carotenoid extract obtained has antioxidant activity with an IC_{50} value of 438 ppm. The resulting IC_{50} value also has an SPF value that falls into the medium category and sunscreen capability as seen from the% Te and Tp value of the sunblock category.

CONFLICT OF INTEREST

The authors declare no conflict of interest

ACKNOWLEDGE

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