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ORIGINAL RESEARCH

Characterization of carotenoid pigments from bacterial symbionts of soft-coral *Sarcophyton* sp. from North Java Sea

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Abstract Marine bacteria produce natural pigments; however, the ability of marine bacterial symbionts to produce natural pigments has been less studied. Marine bacteria associated with soft-coral *Sarcophyton* sp. collected from Karimunjawa Island were successfully isolated and screened to synthesize the carotenoid pigments. This approach has allowed the use of these symbionts as an environmental friendly source of new natural pigments. Out of 33 bacterial isolates, only 4 bacterial symbionts (CBSCP 2-2, CBSCP 2-3, CBSCP 1-1, and CBSCP 2-4), positively contain carotenoid pigments. Molecular identification based on 16S rDNA method showed that bacterial symbionts CBSCP 2-2, CBSCP 2-3, CBSCP 1-1, and CBSCP 2-4 were closely related to *Pseudoalteromonas shioyasakiensis*, *Pseudoalteromonas rubra*, *Virgibacillus salaries*, and *Pseudoalteromonas spongiae*. Pigment analysis showed that the pigments have been categorized within the groups of carotenoid pigments. Antioxidant activity of pigment extracts was done by measuring inhibitory concentration (IC_{50}) of pigment extract against 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution. The antioxidant activity measurement of CBSCP 1-1, CBSCP 2-2, CBSCP 2-3, and CBSCP 2-4 extract, and β-carotene was 2015, 5017, 2520, 4213, and 1980 mg l^{-1}, respectively.

Keywords Bacterial symbionts · Soft coral · Carotenoid · Antioxidant activity

Introduction

The marine environment is the largest habitat on Earth, representing more than 70% of the surface of our planet. These diverse marine environments still remain largely unexplored, understudied, and underexploited in comparison with terrestrial ecosystems and organisms (Joint et al. 2010). Indonesia is the global epicenter of marine biodiversity and is one of the mega diverse countries that harbor the majority of the Earth's species. Indonesia is well known for housing a unique and diverse sponge fauna. Natural products from marine invertebrates greatly expand the chemical diversity available for biotechnological exploitation. The diversity of marine invertebrates has not been utilized optimally, including soft corals (Radjasa et al.

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2007a, 2011, 2013). Variation of the color within soft corals indicates that the organisms have diverse pigment contents, commonly found in yellow, orange, and red colors, which identify the carotenoid pigments.

One of the most serious bottlenecks in developing natural products from marine sources during the past decades has been the availability of biomass and/or of optimised cultivation conditions to gain sufficient amounts of compound for preclinical and clinical studies (Raghukumar 2008; Pan et al. 2008; Schulz et al. 2008). The supply of marine metabolites tested pre-clinically and in the clinic can be provided by several methods, including open aquaculture of invertebrates, total synthesis, semi-synthesis, and fermentation of the producing microbes. Many recognize that fermentation is the most appropriate method for sustainable production of natural products (Salomon et al. 2004). Symbionts that can be cultivated in the laboratory and still produce the bioactive metabolite can then be subjected to enhanced fermentation to produce large amounts of the targeted compound (Hildebrand et al. 2004).

Since marine diversity also reflects chemical diversity, the isolation of the under-exploited bacterial symbionts from Indonesian soft corals offers a great opportunity for discovering novel bioactive pigment compounds based on screening against various disease targets with significant impact and strong potential for treatment of diseases.

The exploitation of symbiotic bacteria as a source for novel secondary metabolites is considered to be in its infancy; however, the discovery rate of novel active metabolites from marine bacterial symbionts could surpass that of their terrestrial counterparts. Among the unusual niches for novel microbes are marine invertebrates, including soft corals, which host hundreds of different bacterial groups and contain diverse symbionts (Radjasa et al. 2007a, 2011).

Carotenoids have important function in photosynthetic process. They play role to absorb light energy in the range of 300–600 nm, beyond absorption area of chlorophyll. The light energy will be transferred to chlorophyll for driving photosynthetic process. Moreover, carotenoids have ability to photoprotect photosynthetic organisms from harmful of excess light during light harvesting in photosynthetic process (Vilches et al. 2011). In biological aspect, carotenoids are well known as an antioxidant and pro-vitamin A to protect the retina from macular degeneration (Dutta et al. 2005). These compounds can inhibit the chain reaction of free radicals that if not inhibited by antioxidants can cause degenerative diseases, such as cancer, coronary heart disease, diabetes, and others (Burrati et al. 2005). Recently, there have been reported novel activities of two specific carotenoids, Siphonaxanthin and Fucoxanthin, which have the potential to inhibit viability of human leukemia cell (Sugawara et al. 2014) and to act as an anti-obesity substance (Gammone and D'Ozario 2015), respectively. In commercial point of view, carotenoids can increase commercial value and quality of many industrial products. Having such potential and great benefits of carotenoids, exploration of the source of carotenoids mainly derived from marine organisms is quite abundant to be exploited.

It has been very well established for more than half a century (Kelecom 2002) that terrestrial bacteria and fungi are sources of valuable bioactive metabolites. It has also been noted that the rate at which new compounds are being discovered from traditional microbial resources, however, has diminished significantly in recent decades as exhaustive studies of soil microorganisms repeatedly yield the same species which in turn produce an unacceptably large number of previously described compounds. Therefore, it is reasonable to expect that exploration of untapped marine microbial diversity and resources will improve the rates at which new classes of secondary metabolites are discovered. In particular, the studies regarding screening of secondary metabolite-producing bacterial symbionts of soft corals are important for understanding their biotechnological potential, including as a sustainable source of marine carotenoids.

Materials and methods

Sampling and isolation of bacterial symbionts

Samples of soft coral were taken from a depth of approximately 2 m by scuba diving from three sampling sites at Cemara Besar, Karimunjawa Islands, North Java Sea (Fig. 1). Upon collection, the soft-coral colonies were then put into plastic containers containing sterile seawaters and then were stored temporarily in a cool box. The samples were rinsed 3× with sterile seawaters to clean the bacteria that temporarily attached to the surface waters (Burgess et al. 2003). The samples were homogenated and were serially diluted. A total of

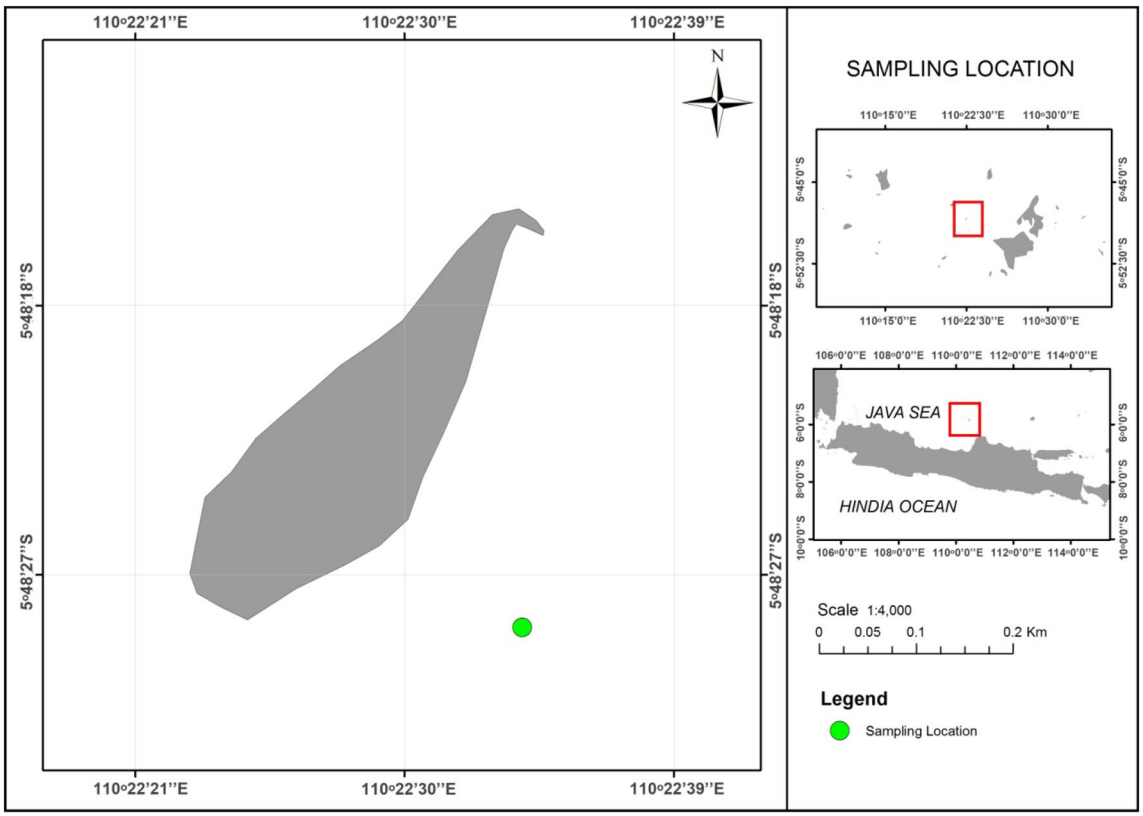

Fig. 1 Location of sampling of soft-coral in Cemara Besar island, Karimunjawa

100 µl were spread on the surface of Zobell 2216E marine medium using glass spreader. Petri dishes were then incubated at 30 °C for 2 days. The appeared colonies with pigment colors were then selected and purified. Unpurified bacteria were picked and streaked into Zobell 2216E medium using four quadrant streaking method. It was incubated at 30 °C for 2 days. A single colony was picked and streaked into new Zobell 2216E medium. The pure isolate was used for further steps.

DNA extraction and 16S rDNA PCR

Genomic DNA of the bacterial symbionts was isolated by the freeze and thaw method (Radjasa et al. 2007b). 1 ml suspension of bacterial cell was centrifuged at 14,000 rpm for 10 min, and supernatant was then discarded. 100 µl nuclease free water was added for resuspending of cell pellet. Bacterial suspensions were frozen at −20 °C and rapidly thawed at 65 °C for 10 min. That freeze and thaw procedure was repeated 4 times and followed by centrifugation at 14,000 rpm for 10 min. Supernatant containing genome DNA was transferred into a new sterile tube. Genomic DNA was used as DNA template in amplification process.

In PCR amplification, primers used for 16S rDNA PCR were universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and Eubacteria-specific primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Long and Azam 2001). The temperature cycle of amplification was as follows: the initial denaturation at a temperature of 94 °C for 2 min, and then successive denaturation (94 °C for 1 min), annealing (55 °C for 1 min), and extension (72 °C for 2 min). A series of denaturation, annealing, and extension were repeated 45 times (Radjasa et al. 2007c). Electrophoresis was done on 2% agarose. Sequencing was done according to Radjasa et al. (2007c). DNA sequencings, homology search, and phylogenetic analyses were carried out according to Atschul et al. (1997) and Radjasa et al. (2013).

Extraction of bacterial pigments

Bacterial symbionts producing pigments were cultured on Zobell 2216E broth medium. They were then centrifuged to obtain bacterial pellet. Pellets were taken and then were extracted using methanol. The extraction process was repeated several times until all pigments were removed and the residues became pale yellow. Pigment extracts were then concentrated using a vacuum evaporator at 30 °C.

UV–Vis spectrophotometer analysis

The extracted pigments were re-dissolved in 10 ml methanol 100%. The spectra were recorded in the λ 300–600 nm range with a Varian Cary spectrophotometer.

Thin-layer chromatography (TLC) analysis

TLC analysis for identification of β-carotene content was done using silica gel GF254 as Stationary phase. Mobile phase used combination of acetone:hexane (6:4 v/v). Retention factor (R_f) value was calculated to determine β-carotene compound.

High-performance liquid chromatography (HPLC) analysis

Carotenoid analysis was carried out by HPLC method. The method involved a Shim-Pack VP-ODS C18 column with methanol:acetonitrile (7:3) as the mobile phase for isocratic elution. Analysis was carried out for 10 min, and flow rates were 1 ml/min. Detection was with a photodiode array detector (PDA). Results of HPLC and spectrum PDA analysis were compared to the literature (Maeda et al. 2005).

Antioxidant activity

Carotenoid pigments from bacterial symbionts and β-carotene marker were dissolved in methanol at several concentrations. Antioxidant activity was measured using spectrophotometer at 517 nm wavelength. Assays were done according to the method reported by Panovska et al. (2005).

DNA sequences

DNA sequences of all bacterial isolates have been deposited in the DNA Database Bank of Japan (DDBJ) in the following accession numbers: NR026223, NR041270, NR043172, and NR125458.

Results and discussion

Results

Screening of bacterial symbionts of soft-coral Sarcophyton sp. resulted in four isolates that are capable of producing pigments. A representative pigment-producing isolates is shown in Fig. 2.

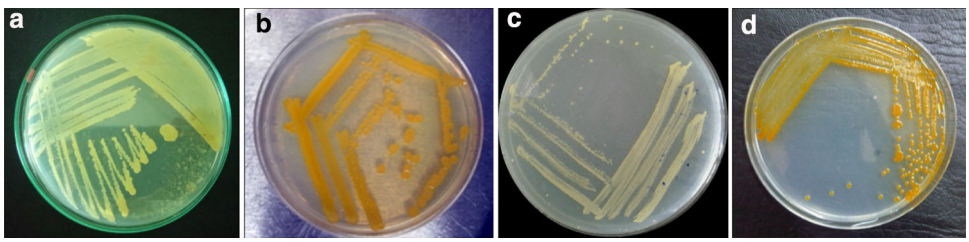

Fig. 2 Bacterial colonies of symbiont of soft-coral *Sarcophyton* sp. **a** CBSCP 2-2, **b** CBSCP 2-3, **c** CBSCP 1-1, and **d** CBSCP 2-4

Table 1 Molecular identification of isolate bacterial symbiont soft coral

No.	Strain	Length (bp)	Closest Relative	Accession number	Homology (%)
1	CBSCP 2-2	1392	*Pseudoalteromonas shioyasakiensis*	LC131142	96
2	CBSCP 2-3	1419	*Pseudoalteromonas rubra*	LC122513	98
3	CBSCP 1-1	1250	*Virgibacillus salarius*	LC131143	92
4	CBSCP 2-4	1393	*Pseudoalteromonas spongiae*	LC131144	97

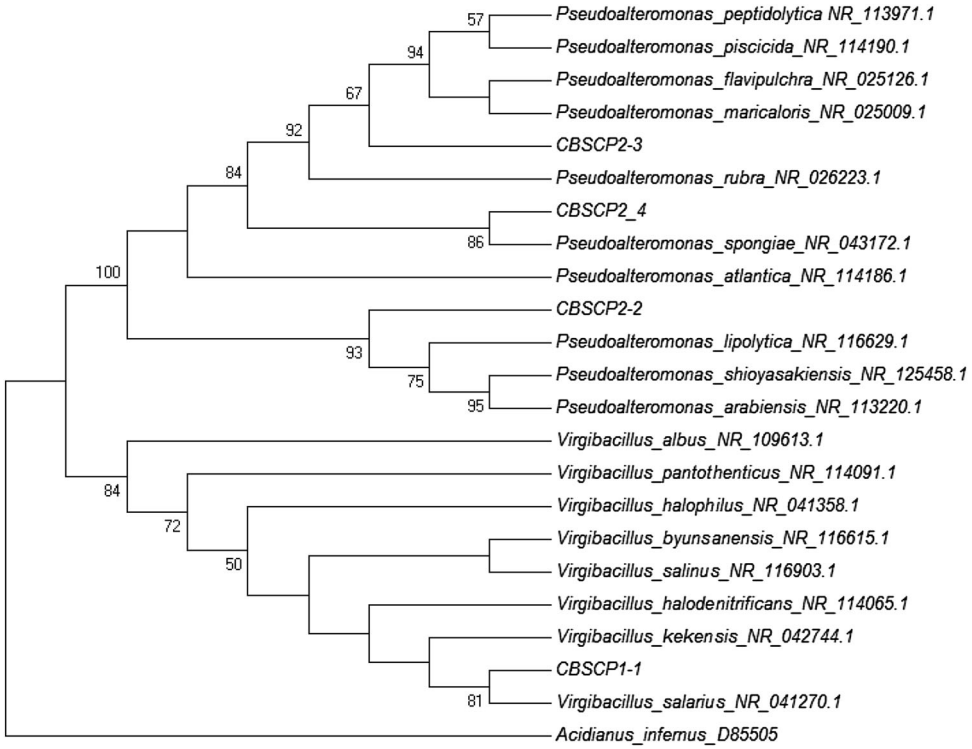

Fig. 3 Phylogenetic tree of pigment-producing bacterial symbionts of *Sarcophyton* sp.

Molecular identification (Table 1) and phylogenetic tree (Fig. 3) reveal that two genera were recognized from four bacterial symbionts, namely, Pseudoalteromonas and Virgibacillus. Three isolates were observed in the genus Pseudoalteromonas, and one isolate was detected in the genus Virgibacillus.

Figure 4 indicates the maximum absorption, which appears on each chromatogram. The results of the spectra obtained at each peak indicate that the pigments produced by all four bacterial symbionts are carotenoids. Bacterial isolate CBSCP 2-2 has three types of carotenoid pigments, isolate CBSCP 2-3 has two to three types of carotenoids, isolate CBSCP 1-1 has two types of carotenoids, and isolate CBSCP 2-4 has one type of carotenoid. Identification for each type of carotenoid produced can be seen from the absorption spectra and the maxima listed in Table 2.

The chromatogram of TLC analyses from β-carotene is shown in Fig. 5. The R_f value of each spot was calculated and then compared with β-carotene marker. The results of TLC have clearly confirmed the results of HPLC, in which the bacterial symbionts contain β-carotene.

The antioxidant activity from pigments produced by each bacterial symbiont is presented in Fig. 6. IC_{50} values from activity antioxidant assay are shown in Fig. 6.

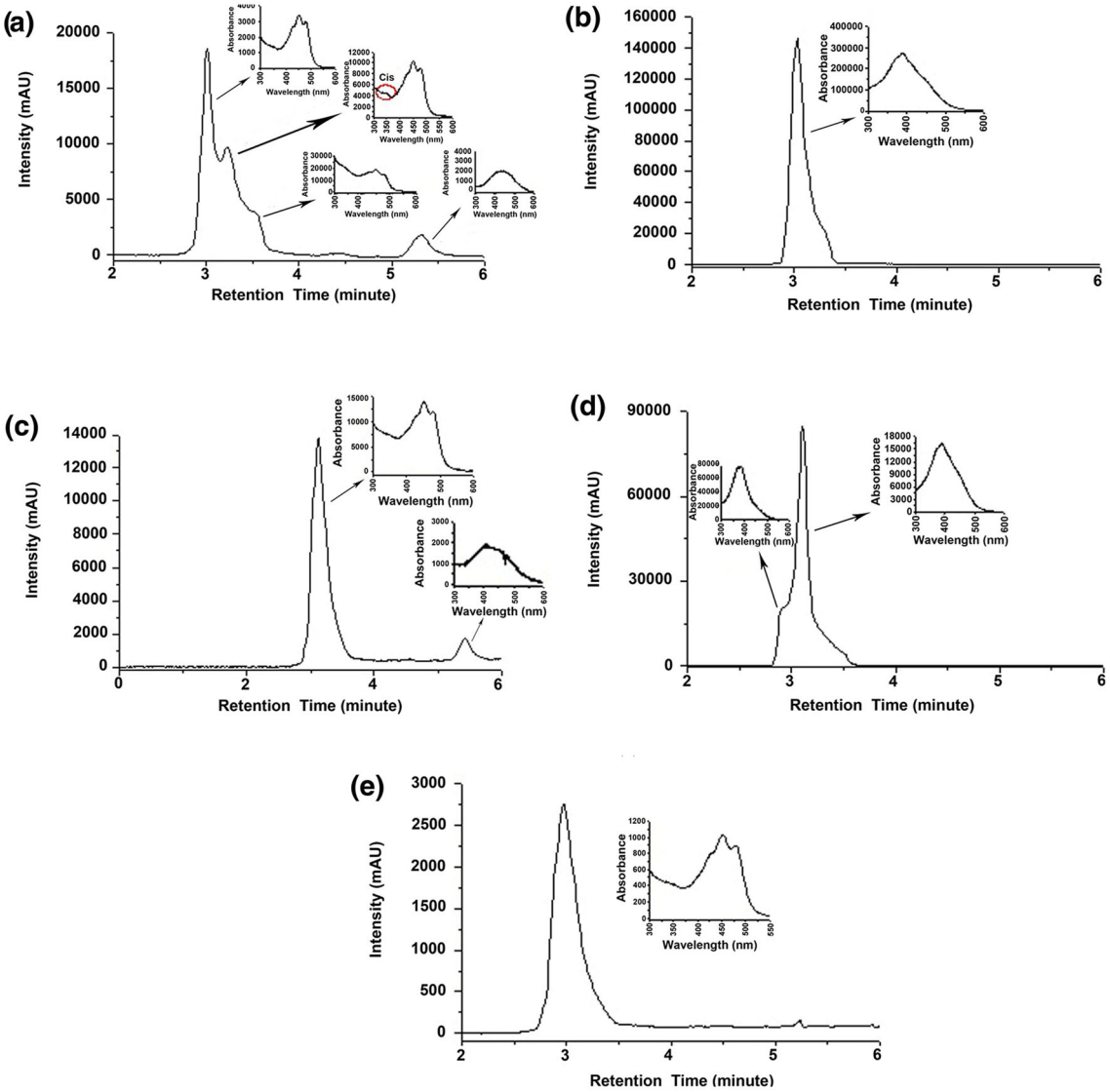

Fig. 4 HPLC profile of the extracts of **a** CBSCP 2-2, **b** CBSCP 2-3, **c** CBSCP 1-1, **d** CBSCP 2-4, and **e** β-carotene marker. The detection was carried out at 450 nm with a flow rate of 1 ml per min^{-1}

Discussion

As an effort to explore the potential of bacterial symbionts of reef invertebrates, especially soft corals, we successfully screened bacterial symbionts of soft-coral *Sarcophyton* sp. collected from Karimunjawa islands in the North Java Sea for producing carotenoid pigments and exhibiting antioxidant activity. The results have prompted the search for novel carotenoid pigment compounds from bacteria associated with soft corals in a sustainable manner, without harming the precious coral reefs. These findings were also in accordance with the highlights of microbial associates of invertebrates with medical potential (Radjasa et al. 2011).

Molecular phylogenetic analysis shows that four bacterial symbionts capable of producing carotenoid pigments are belonging to genera of *Pseudoalteromonas* and *Virgibacillus*. *Pseudoalteromonas* species are frequently found in association with eukaryotic hosts in the marine environment and are well known to produce active metabolites (Holmström and Kjelleberg 1999). *Pseudoalteromonas* is genus that can be divided relatively cleanly into pigmented and non-pigmented species clades and that pigmentation correlates with their proclivity for natural products (Bowman 2007). Furthermore, pigmented *Pseudoalteromonas* species possess a

Table 2 Carotenoid identification of bacterial symbionts of soft-coral *Sarcophyton* sp.

Sample	tr	Component	λ_{max} (nm)			
CBSCP 2-2	2.99	All trans β-carotene		430	451	480
	3.24	Cis β-carotene	(349)	430	451	480
	5.31	Not identified			433	
CBSCP 2-3	3.04	Not identified		371	386	
CBSCP 1-1	3.05	All trans β-carotene		430	451	480
	5.40	Not identified			416	
CBSCP 2-4	2.91	Not identified		370	387	
	3.10	Not identified			389	
β-Carotene marker	2.97	All trans β-carotene		430	451	480

R_f value 0.78

Fig. 5 Result from β-carotene of **a** CBSCP 2-2 and **b** CBSCP 1-1 compared with **c** β-carotene marker by TLC, using silica gel GF_{254} and mobile phase acetone:hexane (6:4)

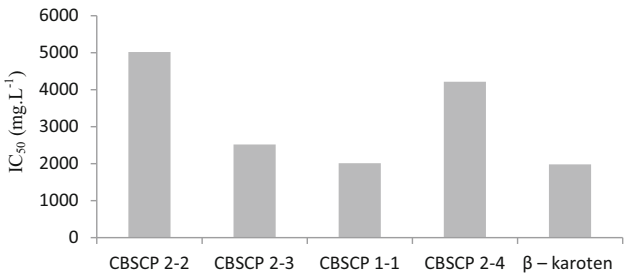

Fig. 6 IC_{50} of pigments of bacterial symbionts CBSCP 2-3, CBSCP 1-1, CBSCP 2-2, CBSCP 2-4, and β-carotene marker using DPPH method

broad range of bioactivity associated with the secretion of extracellular compounds, several of which include pigment compounds (Holmström and Kjelleberg 1999).

On the other hand, one bacterial isolate belonged to the genus *Virgibacillus*. Furthermore, Marhaeni et al. (2011) found that bacterial symbiont *Virgibacillus* sp. from seagrass *Enhalus acoroides* inhibited the growth of biofilm-forming bacteria. A study by Kanagasabhapathyt et al. (2005) demonstrated the inhibition of growth of fouling bacteria by sponge *Pseudoceratina purpure*-associated bacterium *Virgibacillus* sp.

There has been increase in the search for novel pigments especially from bacterial pigments. As an alternative to synthetic pigments, bacterial pigments due to their better biodegradability and higher compatibility with the environment offer promising avenues for various applications. The industry is now able to produce some bacterial pigments for applications in food, pharmaceuticals, cosmetics, and textiles (Venil et al. 2013).

Bacterial symbionts CBSCP 2-2 and CBSCP 1-1 contain carotenoids which have absorptions at 430, 451, and 480 nm. According to Jeffery et al. (1997), carotenoids which have absorptions at a wavelength of 433, 455, and 482 nm are β-carotene.

The chromatogram CBSCP 2-2 shows that there are two β-carotenes. A peak with retention time at 2.99 min^{-1} was identified as all trans-β-carotenes and retention times at 3.24 min^{-1} were identified as cis-isomer of β-carotene by its absorption spectrum: the subsidiary peak in the near ultraviolet (349 nm) is a characteristic for a cis-isomer. The cis-isomer can be identified from the appearance of new peak at 300–375 nm (Gross 1991; Rodriguez-Amaya and Kimura 2004). Identification of all trans-β-carotenes was shown by the similarity of the retention time and Rf value with comparison of β-carotene marker. Judging from the spectrum λ_{max} produced, it is shown that the types of carotenoids produced by bacteria CBSCP 2-3 and 2-4 are almost the same.

IC_{50} concentration of CBSCP 2-2 was 5017 mg l^{-1}, while CBSCP 2-3, CBSCP 1-1, CBSCP 2-4, and β-carotene were 2520, 2015, 4213, and 1980 mg l^{-1}, respectively. β-carotene was used as standard, because it is a natural antioxidant from the carotenoids. IC_{50} value CBSCP 1-1 is in a similar value with β-carotene marker. The smaller the IC_{50} value indicates the higher antioxidant activity.

Arunachalam and Appadorai (2013) reported that an antioxidant activity of bacterial symbiont *Vigibacillus* sp. associated with sponge *Callyspongia diffusa*. Park et al. (2009) also showed the antioxidant activity of pigment-producing marine bacterium *Pseudoalteromonas psicida* TA20.

In conclusion, bacterial symbionts of soft-coral *Sarcophyton* sp. from Karimunjawa islands represent the untapped richness of an under-utilized group of marine microorganisms and the possibility of environmentally friendly secondary metabolite producers with medical potential in particular as antioxidant compounds.

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