Pigments Characterization and Molecular Identification of Bacterial Symbionts of Brown Algae *Padina* sp. Collected from Karimunjawa Island

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Abstract

The search for carotenoids in nature has been extensively studied because of their applications in foods. One treasure of the biopigment source is symbiotic-microorganisms with marine biota. The advantages of symbiont bacteria are easy to culture and sensitize pigments. The use of symbiont bacteria helps to conserve fish, coral reefs, seagrass, and seaweed. Therefore, the bacteria keeps their existence in their ecosystems. In this study, bacterial symbionts were successfully isolated from brown algae Padina sp. The bacterial symbionts had yellow pigment associated with carotenoids. The pigments were characterized using High Performance Liquid Chromatography (HPLC) with a Photo Diode Array (PDA) detector. The carotenoid pigments in the bacterial symbionts were identified as dinoxanthin, lutein and neoxanthin. Molecular identification by using a 16S rRNA gene sequence method, reveals that the bacterial symbionts were closely related to Bacillus marisflavi with a homology of 99%.

Keywords : *carotenoid pigments, brown algae, Padina, bacterial symbionts, 16S rRNA*

Introduction

Pigments are one of the fundamental molecules in photosynthetic organisms. Photosynthetic organisms, including bacteria and fungi, produce phycobilins (phycocyanin, phycoerythrin), chlorophylls (chlorophyll-*a*, chlorophyll-*b*, chlorophyll-*c*), and carotenoids, (*β*-carotene, lutein, fucoxanthin, anthocyanin) (Kong *et al.*, 2010). Carotenoids are well known as an antioxidant and pro-vitamin A to protect the retina from macular degeneration (Steven *et al.*, 2000; Mares *et al.*, 2002; Sashidaran *et al.*, 2013). Carotenoids enhance the immune response and inhibit many types of cancers (Guerin *et al.*, 2003). Carotenoids also help to treat vitamin A deficiency through dietary intake and cancer due to their high-antioxidant activity.

Carotenoids are one class of 800 natural fat-soluble pigments found in plants, algae, and photosynthetic bacteria. Carotenoids can be obtained from many natural sources such as leaves, fruits, coral reefs, seaweed, and fish in terrestrial or marine environments (Sommer *et al*., 1996; Ross, 1999; Olson, 1999). Carotenoids have two main functions in photosynthetic organisms in photo-protection and light-harvesting (Ackleson, 2003; Du *et al*., 2006).

A relatively large amount of heterotrophic bacteria that synthesize carotenoids have been isolated from coastal and oceanic waters (Du *et al*., 2006). However, the widespread occurrence of carotenoids in non-phototrophic bacteria suggests that their presence is crucial for the viability of these organisms in their natural environments (Satfsnes *et al.*, 2010). *Streptomyces, Pseudomona*s, and *Vibrio* are among marine bacteria that produce the bioactive compounds (Jafarzade *et al.*, 2013). Marine bacteria can be an alternative source for exploring marine natural products with promising bioactive compounds. In this preliminary research, the characterization of carotenoid pigments and identification of bacterial symbionts with brown algae *Padina* sp. collected from Karimunjawa Island is reported.

Materials and Methods

Sampling, collection of samples, and bacterial isolation

Colonies of brown algae *Padina* sp. were collected from Menjangan Kecil waters, Karimunjawa islands, Jepara, and North Java Sea, Indonesia by scuba diving. Upon collection colonies

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were put into sterile plastic bags (Whirl-Pak, Nasco, USA). The tissues were then rinsed with sterile seawater and homogenized with mortar. The homogenized tissues were serially diluted, spread on ½ strength ZoBell 2216E, and incubated at room temperature for 48 hours. On the basis of morphological features, 10 colonies were randomly picked and purified by making streak plates (Madigan *et al.,* 2000).

Pigment extraction

Twenty plates of bacterial symbionts of *Padina* sp. cultured on Zobell agar medium were collected. A total of 1 gram of pellets were extracted using cold 100% acetone (Pro Analyst) (Wusqy *et al.*, 2014), with the aid of a sonicator (Britton *et al.,* 1995). The extracted pigments were filtered using filter paper Whatman no. 1. Afterwards, the pigments were then dried by the use of nitrogen (N_2) gas flow.

Identification and analysis of pigment content

Pigments were identified and analyzed by using High Performance Liquid Chromatography (HPLC) Shimadzu LC-20 in reverse phase column AB with ODS, C18, having a diameter of 4 mm x 25 mm (Nugraheni *et al.*, 2010). The mobile phase was comprised of a mixed methanol-acetone solution. The samples were monitored at a wavelength of 190-800 nm with a flow rate of 1 ml / min and a pressure of 1000 psi.

DNA Extraction of bacterial symbiont

The genomic DNA of bacterial symbiont was extracted by using Chelex 100. Bacterial symbiont pellet cells were added with 100 µl of ddH_2O. The mixture was then added with 1 ml of 0.5% saponin in *Phosphate-buffered saline* (PBS) 1X, allowed for an overnight temperature of 4°C. The mixture was centrifuged at 12000 RPM for 10 minutes then 1 ml of PBS 1X was added and centrifuged again at 12000 RPM for 5 min. The mixture was added to 100 µl of ddH_2O and 50 µl of 20% chelex 100. After that, the mixture was boiled for 10 min in hot water with temperature of 95°C, and vortexed once after 5 min. Subsequently, the mixture was run at 12000 RPM for 10 min. The genomic DNA was transferred to a clean Eppendorf tube. The DNA extraction was stored at -20°C.

16S rRNA Polymerase Chain Reaction

Determining the temperature cycle in running PCR followed the following pattern: 94°C for 2 minutes to initial denaturation, 94°C for 1 min to denaturation, 55°C for 1 min to annealing, and 72°C for 2 min to extension respectively. The cycles were repeated for denaturation, annealing and extension until 45 cycles (Murti and Radjasa, 2012). To amplify the 16S rRNA gene, Eubacteria universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), and primer 1492R (5'- TACGGYTACCTTGTTACGACTT-3') were used (Long and Azam, 2001). PCR product visualization was done by using electrophoresis on 2% agarose. The DNA sequencing analysis was done according to Murti and Radjasa (2012).

Results and Discussion

One isolate was selected, namely MKPD3, which was found to produce a yellow pigment and was expected to sensitize one kind of carotenoid.The absorption spectrum of bacterial symbiont was detected from 350–800 nm by using a spectrophotometer UV-Vis (Fig. 2). There were three peaks detected in bacterial symbiont (Fig. 3). As a result, the characterization of carotenoids in bacterial symbiont were dinoxanthin, lutein and neoxanthin (Table 1).

PCR amplification of bacterial symbiont using 16S rRNA showed positive results with the presence of DNA bacterial symbiont with an appropriate base length of approximately 1500 bp (Fig. 4). The phylogenetic tree shown in Fig. 5 was closely related to other microorganisms. By using molecular identification with a two-direction sequencing of the PCR product, it was found that the bacterial symbiont was very close to *Bacillus marisflavi* with 99% similarity (Table 2).

Brown algae are generally known as trash seaweed on shorelines. On the other hand, brown algae have many natural compounds which are useful in many pharmaceutical and food studies. In this study, *Padina* sp. was used as a sample because of its potential in natural bioactive compounds like antioxidants (Setha *et al.,* 2013), antibacterial compounds (Al-Zahrani *et al.,* 2014), and antifungal compounds (Nogueira *et al.,* 2014). Commonly, *Padina* sp. grow abundantly in tropical regions on reef flats and outer reef slopes

Table 1. List of characterization of carotenoid pigments in MKPD3 (Jeffrey *et al.,* 1997; Roy *et al.*, 2011)

No	Retention time (min)	Abs. Peak	Pigment name
1	7.15'	425, 449, 474	Dinoxanthin
2	9.72'	416, 447, 472	Lutein
3	26.52'	417, 438, 467	Neoxanthin

Table 2. Molecular identification of bacterial symbiont

Isolate	Length (bp)	Closest Relative	Homology (%)	Accession no.
MKPD3	918	*Bacillus marisflavi*	99	KJ011883.1

(N'Yeurt and Payri, 2006; Sillberfeld *et al.*, 2013). Morphologically, these algae have a typical fan-shaped thallus, so that it makes the genus *Padina* easily recognizable in the field (Sillberfeld *et al.*, 2013).

Marine algae are classified as green algae (*chlorophyta*), brown algae (*phaeophyta*) and red algae (*rodophyta*) on the basis of chemical composition. It was determined by the color presence of chlorophyll-*a* and -*b*, *β*-carotene (a yellow pigment) and various characteristic xanthophylls (yellowish or brownish pigments) (Chinnadurai *et al.,* 2013). The primary xanthophyll pigment in brown algae is fucoxanthin. Nevertheless, there are other xanthophylls in the *phaeophyceae* class like diatoxanthin, diadinoxanthin, violaxanthin, flavoxanthin, lutein, neoxanthin, tareoxanthin, and violeoxanthin (Strain *et al.*, 1944). From the HPLC data, it was evident that bacterial symbionts produced three xanthophyll pigments consisting of dinoxanthin, lutein and neoxanthin. It was confirmed by Strain *et al.* (1944) that brown algae possess other xanthophyll pigments beside fucoxanthin.

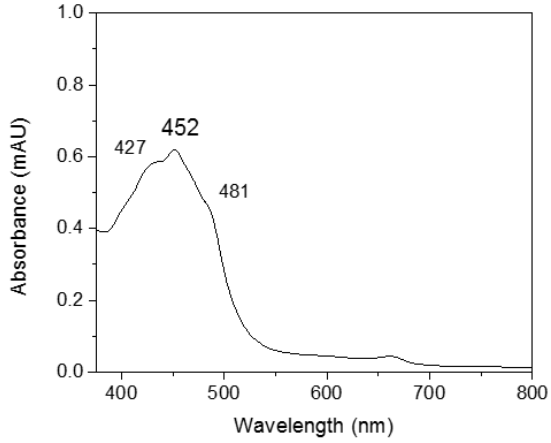

Figure 1. UV-Vis absorption spectrum of MKPD3 crude extracts

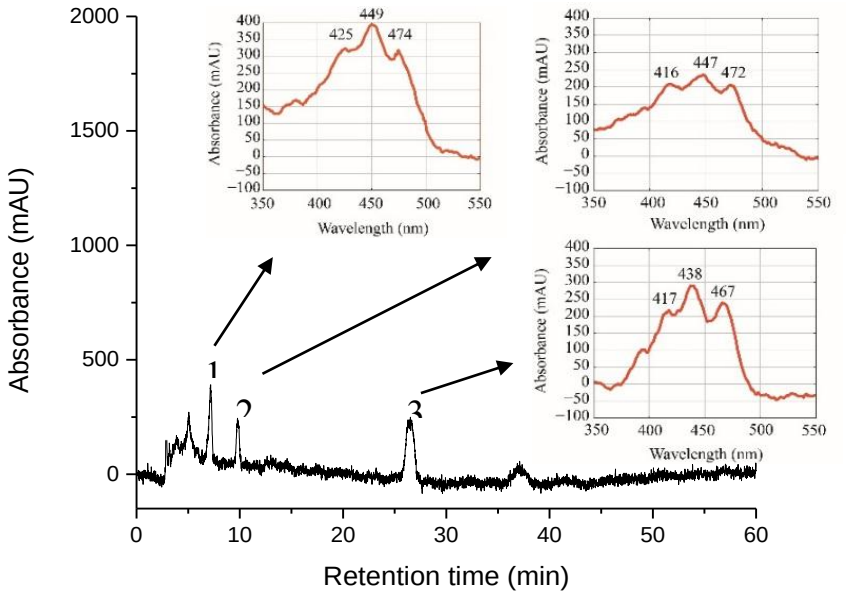

Figure 2. The characterization of carotenoid pigments of MKPD3 analyzed by using HPLC

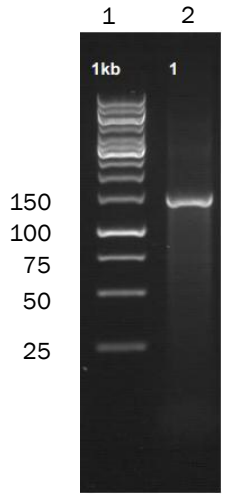

Figure 3. PCR amplification of 16S rRNA fragments. 1: Marker, 2: MKPD3

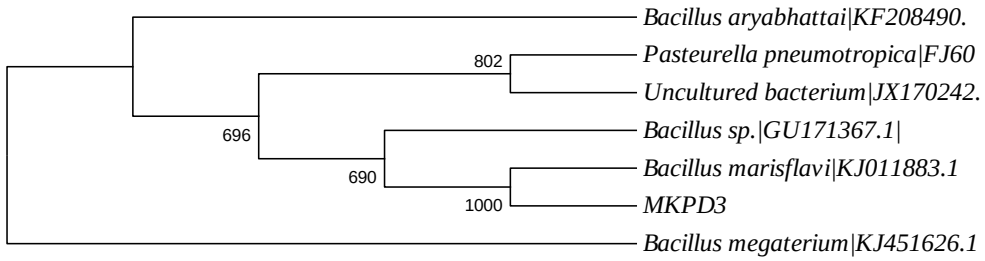

Figure 4. Phylogenetic tree based on 16S rRNA gene sequences of strain MKPD3

Therefore, it is true that symbiont bacteria synthesize similar bioactive compounds like the host.

Several studies on bacterial symbionts have shown that they have potential to function as natural pigment sources and bioactive compounds. Radjasa *et al.* (2008) reported that a marine bacterium *Pseudoalteromonas piscicida* H1.7 isolated from Kakaban Land-Locked Marine Lake showed antibacterial activity towards *Staphyllococcus aureus* and produced a yellow pigment. Another report from Khoeri *et al.* (2011) mentioned that bacterial symbionts of Tunicate *Didemnum molle* with *Virgibacillus* sp. showed antibacterial potential towards MDR bacteria and amplified Non-Ribosomal Peptide Synthetase (NRPS) gene fragments. In addition, Murti and Radjasa (2012) reported that the marine bacterium *Paenibacillus campinasensis* isolated from soft coral *Lobophytum* sp. also showed antibacterial activity towards *E.coli* and *S.aureus*.

Recently, Khaneja *et al.* (2009) successfully identified bacteria from soil, sea water and the human gastrointestinal tract that produced carotenoid pigments. They mentioned that *Bacillus marisflavi* was isolated from soil produced carotenoids. The first report of *Bacillus marisflavi* was proposed by Yoon *et al.* (2003). Gram-positive, endospore-forming, and moderately halophilic rods are the characteristics of this bacteria group. Moreover, *B. marisflavi* has ellipsoidal endospores observed at a subterminal or central position in swollen sporangia and isolated from sea-water of a tidal flat of the Yellow Sea in the Korean Peninsula.

Colonies are pale yellow, smooth, convex, circular to slightly irregular, slightly raised, and 2-4 mm in diameter after 3 days of growth at 30^{o}C on MA. The growth temperature is optimally at 30-37^{o}C. Usually, it grows at 10^{o}C and 44^{o}C, but not at 4^{o}C or above 48^{o}C. The optimum pH for these bacteria is at 6.0-8.0; occurs at a pH of 4.5 and 9.0. In contrast, *B. marisflavi* do not grow at a pH of 4.0 (Yoon *et al.,* 2003). Most species of this genus have carotenoids associated with either the vegetative cell or the spore (Moeller *et al*., 2005; Duc *et al.*, 2006).

Conclusion

The bacterial symbionts of *Padina* sp. have potency as a carotenoids source. Strain MKPD3 contained three essential pigments consisting of

dinoxanthin, lutein, and neoxanthin as shown by HPLC analysis. The bacterial symbionts *Bacillus marisflavi* capable of synthetizing similar pigments to its host organisms. Nevertheless, more developmental research is needed on the applications of carotenoids and bacterial symbionts.

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