CHARACTERIZATION OF CAROTENOID PIGMENTS FROM Sargassum polycystum AND ITS ASSOCIATED BACTERIA

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ABSTRACT

Several bacteria are capable to produce various carotenoid pigments, and they may become potential bioresources for pigment production. Bacteria produce pigments for various reasons and it plays an important role. They may help to protect their host from high exposure of UV-light. However, the ability of marine bacteria to produce natural pigments has been less studied. In this study, we tried to isolate and characterize marine pigment-producing bacteria of brown seaweed macroalgae, Sargassum polycystum from Teluk Awur, Jepara. Out of seven bacterial isolates, only one bacterium, SJ04, positively contains pigments. Pigments analyses using HPLC method showed that pigment composition was slightly different between host and associated bacterium. Pheophorbide a, Fucoxanthin, and β-carotene were detected from S. polycystum as host, whereas Pheophorbide a, and Neoxanthin were detected from the associated bacteria (SJ04). For bacterium identification, molecular genetic approach based on 16S rRNA gene sequence showed that strain SJ04 was genetically closely related to Brachybacterium zhongshanense with 97.97% homology. The opportunity to realize a safe and environmentally friendly with low price, as well as opportunities to find new sources of pigments from bacteria.

Keywords: Carotenoid pigments, Sargassum polycystum, 16S rRNA gene.

INTRODUCTION

Indonesia has abundant natural sources from the sea. One of the nature sources from sea is macro algae or seaweed. Seaweed is one of the most diverse marine biological resources in Indonesia. Based on the records of Van Bosse (through the Siboga Sea expedition in 1899-1900) in Indonesia approximately 555 species from 8642 species of seaweed available in the world. In other words, Indonesia as a tropical region has a seaweed germplasm source of 6.42% of the world's total seaweed biodiversity (Santosa 2003; Surono, 2004). Sargassum polycystum (Agardh 1824) is one seaweed widely consumed. It has the effect of pharmacological properties which will be an important benefit for human health. The present of pharmacological effects depends on the nutrition content such as protein, lipids, carbohydrate, gelatins, chlorophyll, and carotenoid. Carotenoid is organic pigment of yellow, orange, and red color. Carotenoid has function as an antioxidant (Dutta et al., 2005), that would have to remove the free radicals from the system either by reacting with it to yield harmless products or by disrupting free radical chain (Britton 1995).

Carotenoid is also found in microorganisms such as fungi and bacteria. Carotenoid research on bacteria was rarely, especially on marine bacteria. Carotenoid research on bacteria was still slightly, especially marine bacteria. Marine bacteria that have isolated are estimated less than (1 to 2) % of microbial communities which have been successfully isolated from the marine environment as a pure culture. Whereas 98 to 99% of marine bacteria have not succeeded in any culturing techniques. One of the potencies of these bacterial symbionts is as producers of natural pigments, so these can be used as a sustainable source of natural pigments (Krinsky and Johnson 2005). The results of the Nugraheni et al., (2010) study of symbiotic bacteria from the seagrass Thalassia hemprichii produce carotenoids.

Seaweed associated bacteria also has the potential to produce carotenoid pigments. Because seaweed provides a suitable substratum for the settlement of microorganisms and secretes various organic substances that function as nutrients for multi-plication of bacteria (Sigh and Reddy, 2014). Based on the research of Pawar et al., (2015) shows that seaweed produces pigmented bacterial symbionts. Exploitation of new pigment based on marine microorganism is feasible to develop due to the excellence and diversity of marine micro-organism. Exploitation of pigment based on marine micro-organism is very feasible to develop due to the excellence and diversity of marine microorganism.

MATERIALS AND METHODS

Sampling and bacterial isolation: The samples consisted of S. polycystum were taken from Teluk Awur, Jepara, Central Java, Indonesia. Upon collection, the S. polycystum were then put into plastic...
and then stored temporarily in a cool box. The samples were rinsed 3 x with sterilized sea water to clean the bacteria that temporally attached on the surface of seaweed thallus. Isolation bacteria was carried out by the spread method (Radjasa et al., 2007). The sample surface was gently ground and put into tube containing 10 ml sterilized sea water. Subsequently, serial dilution from 10^-1 until 10^-5 was performed using sterilized sea water. From each dilution, 100 µL suspension was taken and spread on the surface of Zobell 2216E marine agar medium to cultivate the associated bacteria. The cultures were then incubated at 37 °C for 72 hours. Bacterial colonies were selected based on color of the colonies. After colored bacterial colonies grew on the surface, the colonies were then purified by using streak method until pure colonies were obtained.

Extraction and characterization of pigments: The pigments were extracted from both S. Polycystum and its associated bacterium to compare composition of the carotenoid pigments. Seaweed sample, S. polycystum was cut into small pieces, and added 1 g of CaCO₃, and then ground using mortar and pastle. The pigments were extracted with acetone:methanol (7:3). Crude pigment extract was dried using nitrogen (N₂) gas. Pigment from the selected bacteria was extracted from bacteria pellet using combination of chemical and physical method. Then was cultured in Zobell 2216E broth medium to obtain sufficient cell pellet for pigment extraction. A total of 5 g of pellets was taken then was extracted using cold methanol, with the aid of sonicator at room temperature in water (Britton, 1995). Extract was centrifuged and filtrate was dried with N₂ gas. Both crude pigment extract from seaweed and its associated bacterium were then analyzed using High Performance Liquid Chromatography (HPLC). The HPLC machine (Shimadzu) equipped with LC-20 in reversed phase column AB with ODS, C18, length 5 m diameter of 4 mm × 25 mm was used for pigment separation. Methanol: acetonitrile with ratio 7:3 (v/v) was used for mobile phase. Flow rate was 1 mL min⁻¹ with pressure 1000 psi. Identification of the carotenoid pigments was analyzed using photodiode array (PDA) detector at wavelength range from 300 to 700 nm. Data of HPLC result were processed with Origin software to identify pigment composition. Furthermore, pigments were identified according to Jeffery et al., (1997) and Zapata et al., (2000).

Antioxidant activity: S. polycystum extract, associated bacteria extract and β-carotene marker was dissolved with methanol at several concentrations. Antioxidant activity was measured using spectrophotometer at 517 nm wavelength. Assays were done according to the method reported by Pringgenies and Idris (2019). The percentage of antioxidant activity was calculated using the formula:

\[
\text{Inhibition} = \frac{[DPPH]_o - [DPPH]_f}{[DPPH]_o} \times 100\%
\]

DNA extraction of seaweed associated bacterium: Genomic DNA of the tested isolate was performed based on chelex extraction protocol. A 100 µl of bacterial suspension was macerated in 1 ml of 0.5% saponin in Phosphate Buffer Solution (PBS) 1X, and incubated overnight at 4°C. Bacterial suspension was centrifuged at 8000 rpm for 10 min, and supernatant was discarded. Cell pellet was washed using PBS 1X, and centrifuged at 8000 rpm for 5 min, supernatant was discarded. Subsequently, 100 µl of 20% chelex 100 in distilled water was added to cell pellet. Sample was mixed well then incubated at 95 °C for 10 min. Subsequently, sample was centrifuged at 8000 rpm for 1 min. Supernatant containing genome DNA was transferred into new 1.5.ml tube and kept at -20 °C. The genome DNA was used for further PCR amplification.

16S rRNA gene amplification and phylo-genetic analyses: In PCR amplification, primers used for 16S rDNA PCR were universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and Eubacteria-specific primer 1492R (5'-TACGYYTACTCTGTTACGACTT-3') (Radjasa et al., 2007). The temperature cycle of amplification was as follows: initial denaturation at a temperature of 94 °C for 2 min, followed by 35 cycles of successive denaturation (94 °C for 1 min), annealing (55 °C for 1 min), and extension (72 °C for 2 min). Final extension was 72 °C for 5 min. The PCR product was analyzed using 1% agarose gel. Sequencing process was done according to Radjasa et al., (2007). Subsequently, Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov) was used for searching identical bacterial species with the tested strain. The phylogenetic inference for sequence data of strain SJ04 was done using the MEGA 7 software (Kumar et al., 2016). A maximum likelihood phylogenetic tree of strain SJ04 was constructed using General Time Reversible (GTR+G+I) model with 1000 bootstrap replications. The sequence data of strain SJ04 was deposited to DNA
Characterization of carotenoid pigments

RESULTS AND DISCUSSION

Sample collection and bacterial isolation: Seaweed sample was successfully collected from Teluk Awur coastal area, Jepara. Morphological characteristics of the sample were brown thallus, small holdfast; forming "y" shaped stolon-like branches, long or oblong form leaf-like thallus, small round vesicles (Figure 1). Those characteristics represented morphological characteristics of Sargassum polycystum. A total 5 different colonies were successfully isolated from S. polycystum. However, only one out of five strains had coloration of the colony (Table 1). Colony of strain SJ04 had yellow coloration (Figure 1), and it indicated that SJ04 was able to produce carotenoid pigments.

Table 1: Characteristics of bacterial strains isolated from S. polycystum from Teluk Awur coastal region, Jepara

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics of bacterial colony</th>
<th>Color</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJ 1</td>
<td>Irregular</td>
<td>White</td>
<td>Convex</td>
</tr>
<tr>
<td>SJ 2</td>
<td>Round</td>
<td>White</td>
<td>Flat</td>
</tr>
<tr>
<td>SJ 3</td>
<td>Round</td>
<td>White</td>
<td>Convex</td>
</tr>
<tr>
<td>SJ 4</td>
<td>Round</td>
<td>Yellow</td>
<td>Convex</td>
</tr>
<tr>
<td>SJ 5</td>
<td>Irregular</td>
<td>White</td>
<td>Rough</td>
</tr>
</tbody>
</table>

Utilizations of associated bacteria of marine organisms are considered as eco-friendly approach for marine natural products explorations, including natural pigments. Their ability to produce unique natural pigment. Using bacteria may avoid over-exploitation of natural resources, and prevent environmental damage (Kusmita et al., 2017). In this study, we successfully isolated pigment-producing bacteria from seaweed S. polycystum using Zobell 2216E marine agar medium. A total five different bacterial colonies can be cultivated in the medium. However only one bacterium, strain SJ04 has yellow coloration of the colony that indicates presence of carotenoid pigment. The visual appearance of color is the initial identification of the presence of pigment in bacteria (Figure 1b). The next step needs to be extracted to find out the type of pigment that exists in these bacteria. Chlorophyll and carotenoid pigments are liposoluble pigments so that solvents can be mixed with water such as acetone and methanol. Acetone is generally used to extract pigments that are non-polar in nature such as: carotene, and methanol is used to extract pigments that are polar such as xanthophyll. Besides these two solvents can also be used to break the bonds of proteins and pigments. The pigment characteristics of these bacteria will be compared with the host.

Extraction and characterization of carotenoid pigments: Result of pigment identification showed slightly different pigment composition between S. polycystum as host, and its associated bacteria. Three pigments were identified from S. polycystum, whereas only two pigments were found from the associated bacteria (Figure 2). From carotenoid group, fucoxanthin and neoxanthin was predominant in S. polycystum and bacterial strain SJ04, respectively.

Figure 1: (a) Seaweed Sargassum polycystum, (b) Bacterial strain SJ04 isolated from seaweed, Sargassum polycystum from Teluk Awur coastal region, Jepara.
Characterization of pigments from *S. polycystum* and associated bacteria was done by using high performance liquid chromatography (HPLC). In the HPLC chromatogram (Figure 2) there were three bands pigments for *S. polycystum* and two bands pigments for bacterial SJ04, chromatogram that can be identified the types of pigments. The identification was conducted by observing the retention time and the spectral pattern produced and they were then compared with literature.

**Table 2:** Pigment identification of typical and main pigments from *S. polycystum* and bacterial symbionts SJ04

<table>
<thead>
<tr>
<th>Peak</th>
<th>Pigment composition</th>
<th>Maximum absorption (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>S. polycystum</em> Isolate SJ04</td>
<td><em>S. polycystum</em> Isolate SJ04</td>
</tr>
<tr>
<td>1.</td>
<td>Pheophorbide a</td>
<td>409, 506, 536, 609, 668</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Phophorbide a epimer</td>
<td>409, 505, 535, 609, 667</td>
<td>408, 504, 534, 608, 666</td>
</tr>
<tr>
<td>3.</td>
<td>Fucoxanthin</td>
<td>449, 469</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>Neoxanthin</td>
<td>413, 439, 468</td>
<td>413, 439, 468</td>
</tr>
<tr>
<td>5.</td>
<td>β-carotene</td>
<td>433, 455, 482</td>
<td>-</td>
</tr>
</tbody>
</table>

The maximum absorption in each chromatogram peak can be used for pigment identification, because each type of pigment has different maximum absorption. The results of the spectra obtained at each peak indicates that the pigment in the bacteria belong to carotenoid because they have maximum absorption around 300 to 600 nm, and chlorophyll derivates because they have maximum absorption around 300 to 700 nm (Gross 1991). The carotenoids content in different bacteria and the host. *S. polycystum* have two and bacterial symbiont have one carotenoid types. The carotenoid from *S. polycystum* can be identified as fucoxanthin and β-carotene. And associated bacteria (SJ04) can be identified as neoxanthin. β-carotene is one of the famous pigments from carotenoid group. It has various kind of biological functions, including as antioxidant substance (Liu et al., 2008), and provitamin A (Olson 1989). The other two pigments, neoxanthin and Fucoxanthin are derivative produc- ts from β-carotene (Dambek et al., 2012). As required from interconversion, neoxanthin and fucoxanthin share the same absolute configuration at C3 and C3’ as C3’S, 5’R and 6’R (Bernhard et al., 1976; Dambek et al., 2012). Neoxanthin is not only a common precursor but also the branch point where the pathway diverges. For conversion of neoxanthin to fucoxanthin, two sequential reactions are necessary: acetylation of an intermediate and ketolation of neoxanthin (Mikami and Hosokawa 2013). Formation of fucoxanthin from neoxanthin involves two modification steps. Apart from the acetylation of the 3’-OH group, a single hydroxylation step at C8 together with the tautomerization of the C7 double bond yields the C8 keto group (Dambek et al., 2012).

**Antioxidant activity:** The DPPH method is often used because it has the advantages of being fast,
selective, sensitive and stable, but also easy to use. The IC50 value determines which conversions work effectively for free radical activity at 50%. The concentration and antioxidant activity are inversely related to the higher IC50 value, the lower the antioxidant activity. The results of the antioxidant activity test are shown in Figure 3.

**Figure 3:** The histogram of IC50 concentration of *S. polycystum* extract, bacterial symbionts (isolate SJ04) extract, and β-carotene using DPPH method

IC50 concentration of *S. polycystum* extract was 2470 ± 3.01 ppm, bacterial symbionts (isolate SJ04) extract was 612 ± 2.11 ppm, and the standard β-carotene as a comparison was 589 ± 2.26 ppm. The concentration between bacterial symbiont extract and β-carotene marker does not differ much, so it can be said to be almost the same.

Carotenoids in bacterial cell membranes have the function of photoprotection by trapping reactive oxygen species produced by sunlight radiation, so they cannot damage the cell membrane (Nishigori et al., 2003; Stahl et al., 2003; Kopsell and Kopsell 2006). The function of photoprotection in the ecology of tropical oceans where sunlight is so strong throughout the year requires adaptation for tropical marine organisms to survive exposure to damaging sunlight. Among the forms of adaptation is to synthesize carotenoids which are responsible for red to yellow, so that the color variations of organisms from tropical oceans are richer when compared to non-tropical oceans.

The function of carotenoids is as an oxygen singlet quencher that can convert singlets into oxygen triplets. The excited carotenoid pigment can release heat then the carotenoid will stabilize. According to Gordon (1990) secondary antioxidants work by binding to an oxygen singlet and converting it to an oxygen triplet. In this mechanism, the antioxidant mechanism of carotenoids can be classified as secondary antioxidants. If seen from its function, carotenoids in *S. polycystum* and associated bacteria (strain SJ04) can also be classified as tertiary antioxidants because they can repair cell damage caused by free radicals (Krinsky 1989). This is consistent with the literature which says that tertiary antioxidants play a role in repairing cell damage caused by free radicals.

**Molecular identification of bacterial strain SJ04:**
A total 1206 bp of 16S rRNA gene sequences was obtained from strain SJ04. Sequence data of strain SJ04 was aligned with 30 bacterial reference data from GenBank. Phylogenetic inference showed that genetically strain SJ04 formed a highly supported clade with other sequence data of genus *Brachybacterium* (Figure 4). Furthermore, BLAST result exhibited that strain SJ04 was genetically identical to *Brachybacterium zhongsanense* with 97.97% homology score (Table 3).
Molecular identification results showed that strain SJ04 has the highest percentage of similarity with *Brachybacterium zhongshanense* with a homology 97.97%. Organisms with 16S rRNA sequence similarity more than 97 % can represent same species (Stackebrandt and Goebel 1994; Hagström et al., 2000; Stackebrandt and Ebers, 2006; Kim et al., 2014) Thus, we conclude that strain SJ04 genetically can be identified as *B. Zhongshanense*. The genus *Brachybacterium* belongs to the family Der- 
mabacteraceae, class Actinobacteria, and was first purposed by Collins et al., (1988) to accommodate *Brachybacterium fæciënum* (Park et al., 2011). Genus *Brachybacterium* are widely distributed in various environments. From marine environments, *B. aquatícum* has been successfully isolated from seawater (Kaur et al., 2016). Zhang et al., (2007) reported that Brachybacterium zhongshanense sp. Nov., cellulose-decomposing bacteria from sediment. So that the possibility of these bacteria has the ability to decompose cellulose from *S. polycystum*. In this study, we also found *B. zhongshanense* associated with seaweed *S. polycystum*. Furthermore, strain SJ04 may become potential bioresources for natural pigment production. However, further application studies particularly in biomass production system are required.

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**Conflicts of Interests**

The author declares no conflict of interests.

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