The Antioxidant Activity of Carotenoid Pigments in the Bacterial Symbionts of Seagrass *Syringodium isoetifolium*

Delianis Pringgenies* and Riyada Idris

Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, Semarang, Indonesia

*Corresponding Authors: pringgenies@yahoo.com (Tel. +62813-9080-0800)

Abstract

Carotenoids are red, yellow and orange pigments which are found in plants, animals, and bacteria, and generally known for their antioxidant activities. This study aims to identify the carotenoid pigments detected in seagrass *Syringodium isoetifolium* bacterial symbionts and know their antioxidant activities. Isolation of bacteria was conducted using a dispersive media Zobell 2116E. Isolated bacterial were cultured and then centrifuged at 8000 rpm for 10 mins and then extracted using methanol. Identification of the pigments was carried out by using High-Performance Liquid Chromatography (HPLC) with reverse phase octadecysil (ODS) column/C18. The mobile phase was a mixture of methanol: acetonitrile (7: 3 v/v). Free radical reduction activities were determined by the method of DPPH (diphenylpicrylhydrazyl) and its absorbance was measured at a wavelength of 517 nm. Identification of the bacterial symbionts from the seagrass *S. isoetifolium* was performed using 16S rDNA PCR method. The results showed that the 12 isolated bacterial were obtained where isolate 7A was found to have carotenoid pigments of peridinin and fucoxanthin. Extracted pigments from the bacteria 7A had free radical DPPH reduction activity of 40%. The results showed that the identification of isolated bacteria 7A had 100% level of kinship with the bacteria *Bacillus amyloliquefaciens*.

INTRODUCTION

Carotenoids are a class of biological pigments which consist of the red, yellow and orange colors. It is one of the pigments with significant potential in healthcare applications. Carotenoids are not inherently manufactured within the biological system of humans and animals, and as such carotenoid intakes are taken from food. Food rich in carotenoids content often come from land animals and plant. However, information on carotenoid-rich marine food sources is still scarce. Arlita et al. in 2013 reported a successful isolation of bacteria *Paracoccus alcophilus* and *Brevibacterium maris* from Caulerpa cupressoides seaweed, which were attributed in producing carotenoids Xanthophyll and carotene [1]. Furthermore, Radjasa in 2003 stated that bacterial symbionts produced pigment similar to that of its host [2]. Based on the findings above, bacteria symbionts can be used as a potential new source for carotenoids because these bacteria are environmentally friendly and can be mass-cultured in a relatively short amount of time. Carotenoid-producing symbiont bacteria can also be found in *Syringodium isoetifolium* seagrass.

Seagrass has a natural hue of green to yellowish green, yet there has not been much information written on the symbiont bacteria which produced pigments of these colors. This research aims to identify carotenoid pigments from symbiont bacteria of seagrass *Syringodium isoetifolium* as antioxidants.

EXPERIMENTAL

General

Samples of *Syringodium isoetifolium* seagrass were collected from Teluk Awur, Jepara, Indonesia. The extracts from bacterial extraction were analyzed using UV-Vis CARY 50 in the range of 190 – 800 nm. High-Performance Liquid Chromatography (HPLC) was used on a model of Shimadzu LC 20-AB consisting reversed phase octadecysil (ODS) column/C18 (ODS/C18, 4 mm x 25 mm diameter) with 5 µL injection, methanol:acetonitrile (7:3 v/v) as a mobile phase and flow rate of 1 mL.min⁻¹ and 1000 psi of pressure [4]. The analysis was then carried out at 190 - 800 nm wavelength. HPLC results were analyzed using OriginPro 8.1 software.

Sample Collection and Isolation of Symbiont Bacteria

Seagrass samples in 5 grams were directly placed within sample tube. The tube was prepared prior to sample collation by adding seawater and storing in cool box. The samples were then...
cleaned-off from bacteria with sterile seawater. The samples were then digested and were mixed into 5 mL of seawater. This process yielded 10^4 of sample dilution. Diluted sample, 0.5 mL was taken off and transferred into a reaction tube with a sterile pipette. The reaction tube was prepared before by adding 4.5 mL of sterile seawater. This process yielded 10^1 of sample dilution. The processes were repeated until sample dilutions of 10^2, 10^3, 10^4, 10^5, and 10^6 were obtained. Of each sample dilution factor, 100 μL of sample was dispersed over Zobell 2216E media by using a spreader and all of the samples were incubated for 3 days in 30 °C of temperature [2]. Colonies displaying hues of yellow and orange (5.A.4) were selected and purified.

Identification of Symbiotic Bacteria

Obtained DNA from the 24-hours bacterial cultures were extracted using the High Pure PCR Template Preparation Kit (Roche). PCR 16S rDNA amplification was carried out by denaturation at 94 °C for 5 mins as initial heating, followed by 30 cycles (annealing at 94 °C for 30 s, extension at 54 °C for 60 s and renur of denaturation process at 72 °C for 120 s) and incubation at 4 °C. The primer used in PCR 16S rDNA process is universal primer 27F (5’-AGAGTTTGATCMTGGCTCAG-3’) and eubacteria-specific primer 1492R (5’- TACGGYTACCTGTAGACTT-3’) [3]. The electrophoresis process was agarose gel with 1% concentration. The device was operated at 100 V for about 45 mins. The obtained result from the electrophoresis process was observed under a UV Illuminator. The sequencing process was carried out in compliance with PCR sequencing cycle using Big Dye Terminator v.3.1 and the DNA sequences were compared with sequences in DNA database at Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information, National Institute for Health, USA (www.ncbi.nlm.nih.gov).

Bacterial Culture and Extraction

Bacterial culture to obtain bacterial biomass was carried out in several phases using liquid Zobell 2216E. The bacteria were harvested and put into centrifuge until pellet was obtained, after that the weight of pellet is measured. The obtained pellets were diluted in methanol to separate bacteria and its pigments and then fixated by Nitrogen gas to obtain raw extracts. The obtained bacterial raw extracts were then dry-weighed before being put into another dilution process using methanol.

DPPH Test

DPPH test for bacterial pigment samples employed in a similar manner with previous method with several modifications [5]. This method was performed by preparing 3 mL of DPPH stock 0.1 mM and added into 1 mL of test pigment solution (2000 ppm concentration). The control of extract was replaced by methanol, incubated for 30 mins in a dark room. Absorbance rate was then calculated by using spectrophotometer set at maximum wavelength for DPPH (517 nm). Inhibition rate was then calculated by using spectrophotometer set at maximum wavelength for DPPH (517 nm). Inhibition rate calculation was also performed on the processed pigment samples.

RESULTS AND DISCUSSION

Identification of Bacteria

Figure 1 shows DNA amplification with PCR 16S rDNA in which gave positive results with displayed DNA files from the bacteria at the length of > 1000 bp. The primer used in this PCR 16S rDNA amplification was universal primer 27F and eubacteria-specific primer 1492R. Sequencing result was displayed in the form of base sequence of isolate 7A (>1000 bp) was displayed in Figure 2.

The resulting sequences were then matched with the sequences in the database bank. The search was performed on internet at BLAST, USA. The analysis results of the phylogenetic tree for isolate 7A bacteria was shown in Figure 3. Homology tracing results for isolate 7A with BLAST searching were listed in Table 1.
Homology tracing results for isolated 7A showed that the *Bacillus amyloliquefaciens* in the isolate matched 100%. Bacteria of the genus *Bacillus* are categorized as probiotic [6], which are non-pathogenic microorganisms acting as a microbial balancer in digestive system through competitive exclusion. Bacteria of genus *bacillus* are gaining interest in human health-related functional food research couple with their enhanced tolerance and survivability under hostile environment of the gastrointestinal tract [7].

Recently, probiotics are widely used as additives for both human and animal food. Probiotics are beneficial in that they contribute to acidity regulation and inhibit the growth of pathogenic bacteria in the digestive system. In addition, *Bacillus* is one of the bacteria capable of producing various enzymes where it can be used for breaking down substances like carbohydrate, fat, and protein into simpler substances, allowing easy nutrition absorption by chickens. Wizna et al. (2007) found that cellulolytic bacteria *Bacillus amyloliquefaciens* isolated from the forest peat moss litter in Lunang, Pesisir Selatan District, West Sumatera which was described as Gram-positive, rod-shaped, elliptical endospore producing, displayed transparent zone on medium CMC 27.85 mm and cellulose enzyme activity Cx and C1 in high-fiber medium (24%) as much as 0.488 and 1.200 U/mL [8]. Diaz (2007) reported that the use of *Bacillus amyloliquefaciens* CECT 5940 as probiotic additive in dietary intake for broilers at the dose of 1x10^9 cfu/kg yielded a diet conversion of 1.84 [9].

### Identification of Pigment

The analysis result of *Syringodium duplicatum* (7A) seagrass raw extract using HPLC was displayed in Figure 4. The absorption spectra from chromatogram of the analyzed pigment on coral symbiont bacteria (7A) can be seen in Figure 5. Maximum absorbance of HPLC analysis on components of the extracts of coral symbiont bacteria (7A) is presented in Table 2.

#### Table 1. Homology tracing results from BLAST searching

<table>
<thead>
<tr>
<th>Homology tracing results</th>
<th><em>Bacillus subtilis</em> strain yxw4 16S ribosomal RNA gene</th>
<th><em>Bacillus subtilis</em> strain 2B 16S ribosomal RNA gene</th>
<th><em>Bacillus amyloliquefaciens</em> strain 1A 16S ribosomal RNA gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max score</td>
<td>2536</td>
<td>2536</td>
<td>2536</td>
</tr>
<tr>
<td>Total score</td>
<td>2536</td>
<td>2536</td>
<td>2536</td>
</tr>
<tr>
<td>Query cover</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>E value</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ident</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Accession</td>
<td>KF278950.1</td>
<td>KF112078.1</td>
<td>KF112077.1</td>
</tr>
</tbody>
</table>

### Identification of Pigment

Figure 4. Chromatogram profile of raw extract of coral symbiont bacteria (7A) displayed 3 dominant peaks.

Figure 5. Spectrum pattern from pigment analysis on seagrass symbiont bacteria (7A) with (a) 3.52 mins retention time, (b) 6.03 mins retention time and (c) 6.65 mins retention time.

#### Table 2. Maximum absorbance from HPLC of seagrass symbiont bacteria raw extract component (10.B.2)

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Retention Time (Mins)</th>
<th>Components</th>
<th>Maximum Absorbance (nm)</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.52</td>
<td>Peridinin</td>
<td>469</td>
<td>47*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6.03</td>
<td>Fucoxanthin</td>
<td>448</td>
<td>44*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6.05</td>
<td>Unknown</td>
<td>404</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Data processing on Origin 8.1 software displayed in different spectrum patterns for each chromatogram peak. The carotenoid pigment was identified based on the spectrum pattern of each peak and its polarized order in comparison with previously reported results [10] which utilized relatively similar solution composition and static phase for studying phytoplankton. HPLC analysis produced one peak (Peak No. 3) with a maximum absorbance rate of 404 nm which cannot be identified for pigments. This is due to the fact that the corresponding retention time and maximum absorbance rate of the peak did not match any of the categories in the reference data. However, it is believed that the peak may correspond to a pigment belonging to xanthophyll class. Peak 1 with 3.52 mins of retention time and maximum absorbance at 469 nm wavelength highly match with the pigment Peridinin. Peak 2 with 6.03 mins of retention time, and maximum absorbance at 448 nm wavelength was found to be the pigment Fucoxanthin. Fucoxanthin is a member of carotenoid pigment xanthophyll and is often found as primary pigment in many species of brown algae. Fucoxanthin acts as an active biological component, of which one of the benefit is anti-obesity. Fucoxanthin has shown to possess the capability to stimulate the liver to produce DHA, although the mechanism of this action has not yet been fully understood. Fucoxanthin has been shown to be potential in applications to treat obesity as a very potential food supplement [11].

**CONCLUSION**

The research found that the symbiont bacteria of *Syringodium isoetifolium* seagrass was *Bacillus amyloliquefaciens*. By using HPLC and UV-vis measurements, it was confirmed that the bacteria produced Fucoxanthin as one of the carotenoid pigments. The isolate 7A with such pigments showed capacity in inhibiting free radical of DPPH up to 40%.

**ACKNOWLEDGEMENT**

The authors thank to the Head of the Natural Drugs Laboratory of Diponegoro University and Head of Chemistry Laboratory of Universitas Kristen Satya Wacana who has provided research facilities.

**REFERENCES**


**Abstrak**

Karotenoid merupakan pigmen merah, kuning dan orange yang ditemukan dalam tanaman, hewan dan bakteri, serta diketahui memiliki aktivitas antioksidan. Penelitian ini bertujuan untuk mengidentifikasi pigmen karotenoid yang terdapat dalam bakteri simbion lamun *Syringodium isoetifolium* dan mengetahui aktivitas antioksidannya. Isolasi bakteri dilakukan dengan metode sebar pada media Zobell 2116E. Isolat bakteri dikultur dalam media cair Zobell 2116E dan kemudian disentrifugasi dengan kecepatan 8000 rpm selama 10 menit dan diekstraksi menggunakan pelarut metanol. Identifikasi pigmen dilakukan dengan menggunakan Kromatografi Cair Kinerja Tinggi (KCKT) fase terbalik ODS/C18. Fase gerak adalah campuran metanol:asetonitril (7:3 v/v). Aktivitas peredaman radikal bebas ditentukan dengan metoda DPPH (difenylpicrilhidrazyl) dan absorbansiya diukur pada panjang gelombang 517 nm. Identifikasi pigmen simbion lamun *S. isoetifolium* dilakukan dengan metode PCR 16S rDNA. Hasil penelitian menunjukkan bahwa 12 isolat bakteri yang diperoleh dimana isolat 7A ditemukan mempunyai pigmen karotenoid piridinin dan fukosantin. Ekstrak pigment dari bakteri 7A memiliki aktivitas peredaman radikal bebas DPPH sebesar 40%. Hasil tersebut menunjukkan bahwa identifikasi dari bakteri isolat 7A memiliki tingkat kekerabatan sebesar 100% dengan bakteri *Bacillus amyloliquefaciens*.

**Kata kunci:** aktifitas antioksidan, lamun, pigment karotenoid, *Bacillus amyloliquefaciens*. 