

Original Paper

CHARACTERIZATION OF CAROTENOID PIGMENTS FROM BACTERIAL SYMBIONTS OF SEAGRASS *Thalassia hemprichii*

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ABSTRACT

Carotenoids are pigments that can be used in various applications including cosmetics and precursor of vitamins A. Carotenoids are mostly found in higher plant leaves, fruit, and bacteria. Marine bacteria associated with seagrass Thalassia hemprichii collected from Menjangan Kecil Waters, Karimunjawa Islands were screened to produce the pigment and has allowed the use of these microorganism as an environmental friendly alternative source of new natural pigment. The isolation of bacterial symbionts on Zobell 2216E medium from seagrass Thalassia hemprichii resulted in 20 isolates of which 8 bacterial symbionts have produced pigments but only one bacterium positively synthesize carotenoids. Initial analysis with atomic absorption spectrophotometric method revealed that the wave length of bacterial pigment were in the range of 300-600 nm, which are categorized that within the group of carotenoid pigments. From the results of molecular identification by 16S rDNA method, it was shown that bacterium TH8 was closely related to Bacillus licheniformis with 98% homology value.

Keywords: *Thalassia hemprichii* ; carotenoids ; HPLC; Diadinoxanthin ; 16S rDNA PCR.

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INTRODUCTION

One of the most important Indonesian natural resources that have a significant role is natural pigments, such as the green chlorophyll, carotenoids, yellow-red, yellow curcuminoids, as well as anthocyanin that gives different colors, like red, blue and purple. Natural pigments have many functions both in the process of photosynthesis of plants, and in the production of secondary metabolites.

Carotenoids also play important roles for health and human survival. Carotenoids are believed to improve the better immune responses, protection from cancer and also function as an antioxidant. Children who are malnourished often have lower concentrations of serum carotenoids compared with good nourished children. Carotenoids can function as antioxidants (Santamaría *et al.*, 1988), anticancer (Mathews-Roth, 1987; Temple and Basu, 1988) and also used in the treatment of

diseases that are sensitive to light (Mathews, 1964). Carotenoids are yellow pigments, orange to red. pigments and are found in vegetables and fruits, and found also in fungi, bacteria, animals and humans (Gross, 1991).

Microbes that have been isolated from the marine environment are estimated less than 1-2% as a pure culture, whereas 98-99% have not succeeded in any culturing techniques. Similarly, the data on diversity of bacterial symbionts in the seagrass is also still very limited. It has been reported that there are microorganisms associated with marine organisms is also suspected to synthesize secondary metabolites similar to their host (Watermann, 1999). Microbes isolated from plants, which produce bioactive substances have been found to have greater activity, even greater activity than the activity of the host plants. One of the potential of these bacterial

symbionts is producing a natural pigment, so these organisms can be used as a sustainable source of natural pigments (Krinsky and An, 2005).

Symbiotic marine bacteria with this seagrass, also has the potential to produce natural pigments. Exploitation of new pigment-based on marine microorganisms is very feasible to develop due to the excellence and diversity of marine microorganisms, the opportunity to realize a safe and environmentally friendly pigment is affordable, as well as a chance discovery of a new type of pigment that is useful.

MATERIALS AND METHODS

Sampling and isolation of bacterial symbionts

Samples of seagrass *Thalassia hemprichii*, were taken from a depth of approximately 2 m by using a cutter, which were then put into sterile plastic bag and then were stored temporarily in a cool box. The samples were rinsed 3x with sterile sea water to clean the bacteria that temporarily attached to the surface waters. Furthermore, the sample surfaces were scraped and spread on the surface of Zobell 2216E medium with a sterile cotton bud (Burgess *et al.*, 2003). Petri dish was then incubated at 30°C for two days (Radjasa *et al.*, 2007). The colonies with colored pigments were then selected and purified.

Pigment extraction bacteria

Bacterial symbionts were cultured on Zobell broth medium, which were then centrifuged for bacterial pellet. A total of 1 gram of pellets were taken and then were extracted using cold acetone-methanol (7:3 v / v) (Cohen-Bazire *et al.*, 1957; Kuki *et al.*, 1994), with the aid of sonicator (Britton, 1995).

Identification and analysis of pigment content

Pigments were identified and analyzed by using High Performance Liquid Chromatography Shimadzu LC-20 in reversed phase column AB with ODS, C18, 5 m. diameter of 4 mm x 25

mm and a mobile phase of methanol: acetonitrile. Detection of pigment was performed at a wavelength of 190-800 nm with a flow rate 1 ml / min, pressure 1000 psi (Maeda, 2005).

16S rDNA Polymerase Chain Reaction

The primers used for 16S rDNA PCR were Eubacteria universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Long and Azam, 2001). The temperature cycle of amplification was as follows: 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 minutes). Series of denaturation, annealing and extension were repeated until 45 cycles (Radjasa, 2005b). Electrophoresis was done on 2% agarose. Sequencing was done according to Radjasa *et al.* (2007a). Homology search and DNA data bank by BLAST (Atschul *et al.*, 1997).

RESULTS AND DISCUSSION

Sampling and isolation of bacterial symbionts

Thalassia hemprichii samples used in this study were taken from Menjangan Kecil Waters, Karimunjawa Islands (Fig. 1).

Of the 20 isolates (Table 1) 8 isolates were found to produce pigments, but only one bacterium TH8 (Table 1) contains carotenoids.

Pigment extraction of bacterial symbiont

A total of 200 gr wet weight were obtained from the TH8 isolate (Fig. 3) which were extracted, resulted in 5 grams of red pigments.

Identification and analysis of pigment content

Results of identification of pigments by HPLC method is presented in Fig. 4.



Fig.1. Samples of seagrass *Thalassia hemprichii*

The successful isolation of bacteria in 20 isolates associated with seagrass culture media on Zobell 2216E (**Fig. 2**) resulted *Thalassia hemprichii*.



Fig. 2. Bacterial symbionts of the seagrass *Thalassia hemprichii*

Table 1. The bacterial symbionts of seagrass *Thalassia hemprichii*

No.	Bacteria Code	Color	Form	Texture
1	TH1	Yellow	irregular	Coarse
2	TH2	White	small round	Coarse
3	TH3	Transparent white	irregular	Concave
4	TH4	Transparent white	irregular	Coarse
5	TH5	Yellowish white	Round	Convex
6	TH6	White translucent	Round	Smooth
7	TH7	Milky white	Round	Convex
8	TH8	Pink	Round	Convex
9	TH9	Milky white	small round	Convex
10	TH10	Yellow translucent	Round	Convex
11	L2S1	Yellow	small round	Convex
12	L2S2	Yellow	round	Convex
13	L2S3	Yellow translucent	irregular	Smooth
14	L2S4	White translucent	irregular	Coarse
15	L2S5	Yellow translucent	Round	Cekung
16	L2S6	Yellow translucent	small round	Convex
17	ENS1	Yellowish white	Round	Convex
18	ENS2	Yellow	Round	Convex
19	ENS3	Yellow	Small rund	convex
20	ENS4	Yellow translucent	irregular	coarse



Fig. 3. Pigment extract of bacterium TH8 obtained from Zobellbroth medium

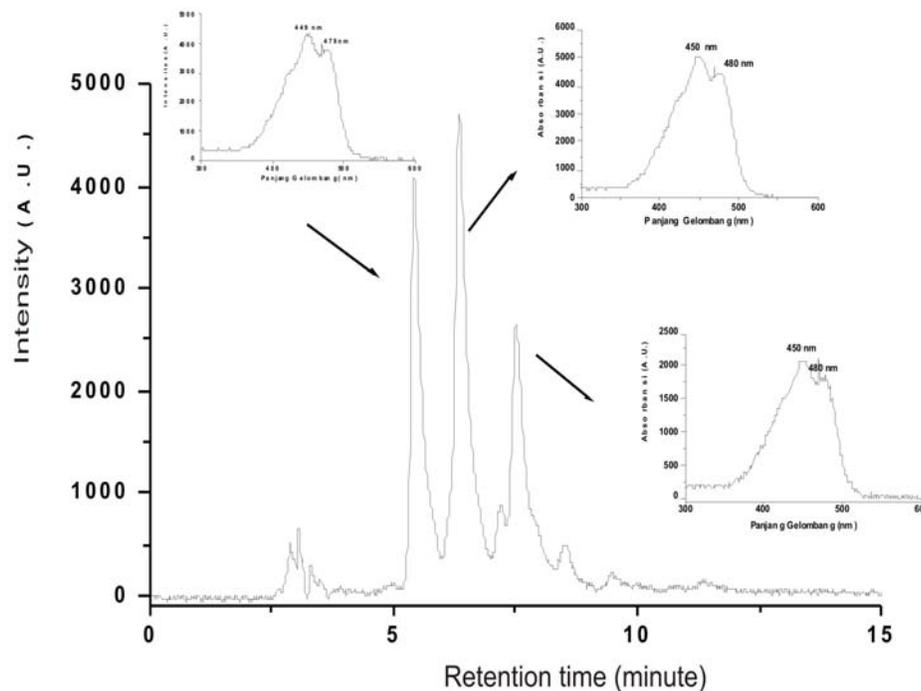


Fig. 4. Chromatogram and spectra of the bacteria TH8 with 449nm wavelength and two wavelengths of 450nm

Results of analysis showed the presence of three peaks, in which each peak was an indication of the presence of compounds. In the HPLC chromatogram (Fig. 4) there were three bands pigments that can be identified from HPLC Identification of pigments was done by analysis of spectra in the band pattern of the chromatogram. Pattern spectra are presented in (Fig. 5). Based on the area of pigment it can be determined percentage of pigment content. The

percentage of pigment content are presented in Table 2.

Results of spectrophotometer with methanol acetone reagents show that the spectral pattern formed has a peak absorption at a range of wavelength between 300-500 nm at visible light. This shows that the pigments contained in the bacterium TH8 are carotenoids, because carotenoids have a maximum absorption at a wavelength of 300-600 nm (Gross, 1991). Carotenoid pigments are

distinguished into two groups, namely carotenes such as those found in carrots (*Daucus carrota*) and xanthophylls (yellowing of leaves) (Gross, 1987; Gross, 1991; Britton *et*

al., 1995). Carotenoids of xanthophyl groups are used by photosynthetic organisms and essential for the defense of the organism (Eonseon *et al.*, 2003).

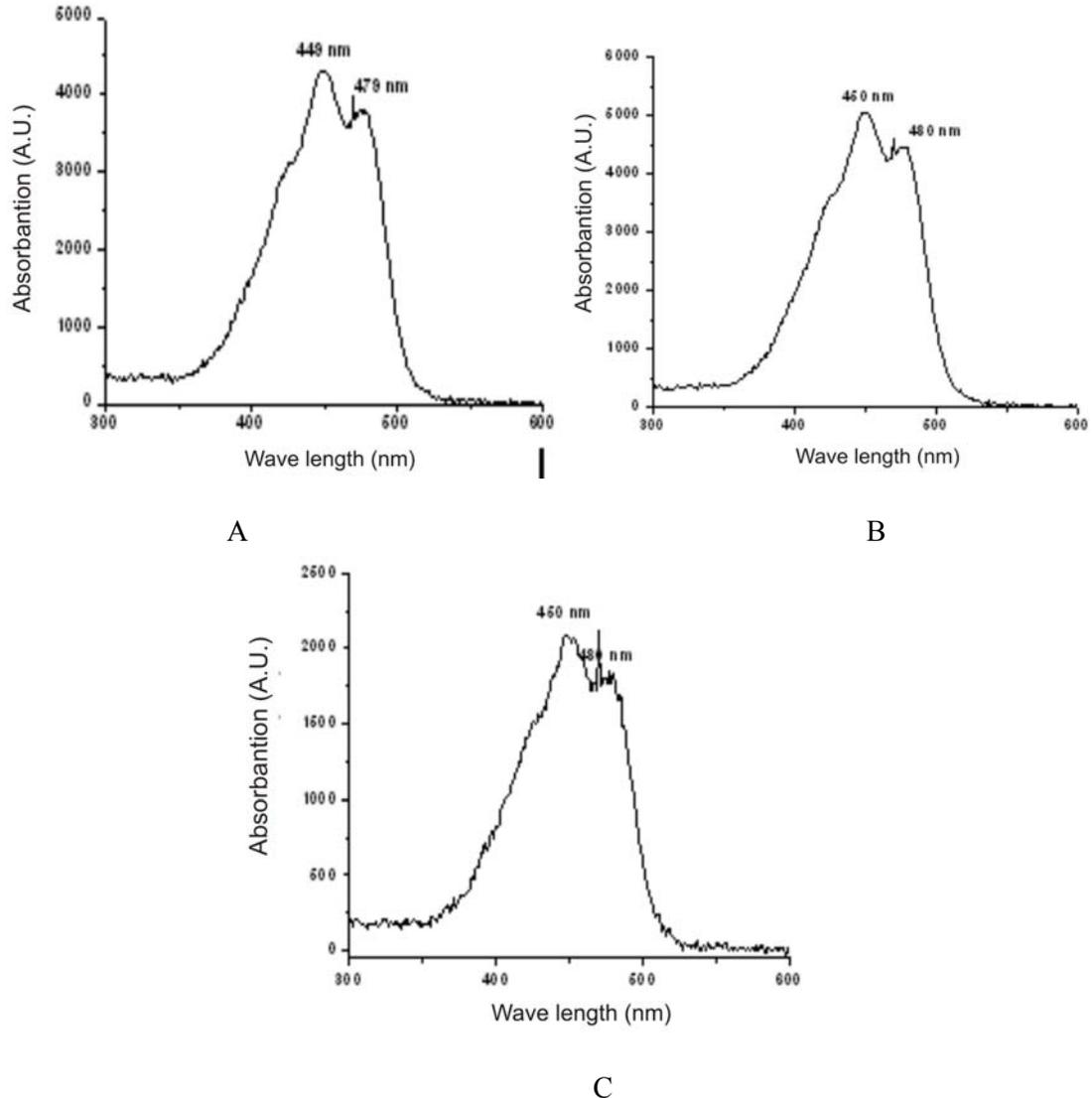


Fig 5. The spectra of HPLC pigment pattern of Bacterium TH8 at three different wave lengths: A.449 nm, B. 450 nm, C. 450 nm

Table 2. Percentage of pigment content of bacterium TH8

Ribbon	Type of Pigment	% Content
1	Trans-Diadinoxanthine	40,05%
2	Cis-Diadinoxanthine	11,31%
3	Isomer Diadinoxanthine	12,87%

From the results of HPLC analysis on the absorption wavelength of 449nm and 450nm showed that the bacterium TH8 associated with seagrass *Thalassia hemprichii* has spectral

pattern of pigment-like shape pattern spectra of diadinoxanthine class of carotenoid pigments (Jeffrey, 1997). The molecular structure of diadinoxanthine presented in **Fig. 6** below.

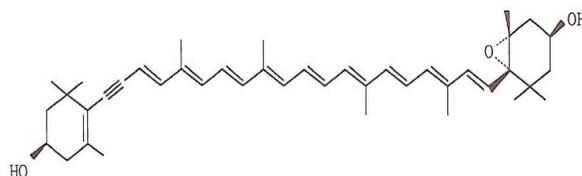


Fig. 6. Molecular Structure Diadinoxanthine (Jeffrey 1997)

Diadinoxanthines are compounds with chemical structure $C_{40}H_{54}O_3$, pigment is included in xanthophyl group I that normally found in diatoms and dinoflagellata. Diadinoxanthin included in the xanthophyl group because it has oxygen in ring ionon. Therefore it can be concluded that the class diadinoxanthine found as carotenoids of the TH8 isolate is a carotenoid of xanthophyl class.

From the results of HPLC analysis for the three peaks (**Table 2**) have a maximum absorption patterns that almost the same. The differences of the three peaks are only in stereoisomer which is a compound that has the same molecular formula. Carotenoids are compounds that have a lot of double bonds in there structure, that caused the phenomenon of carotenoid cis-trans isomerization (Zechmeister, 1962). A cis-double bond implies a configuration with these groups the highest priority on the same side, while on their trans configuration on the opposite side. The number of stereoisomers that may be of a product is very high, depending on the number of double bonds in the molecules.

According to Gross (1991) in natural carotenoid is found more often in the form of trans isomers, trans isomers form changes into the cis isomer is due to some natural factors such as: rising temperatures, increased light intensity and the effect of differences in salt content. Therefore, the bond trans content is higher than the cis bond. Several studies previously reported diadinoxanthine pigments contained in the diatom and dinoflagellate, while for diadinoxanthine that are present in bacterial symbionts of plants, seagrasses have

not been reported. Previous studies on bacterial symbionts of seagrass have been reported as antibacterial pathogens (*Staphylococcus aureus* and *Escherichia coli*) and fungal pathogens (*Candida albicans* and *Aspergillus niger*). While for the bacterial symbionts that contain carotenoids seagrasses have not been found so far (Kurniawan *et al.*, 2009)

From the research results diadinoxanthine pigment confined to the identification and diadinoxanthine cycle (Jeffrey, 1997). Utilization of derivatives of xanthophylls far are astaxanthine which has been found in plants and algae, lutein and zeaxanthine contained in egg yolk. Astaxanthine itself has many biological functions such as for photoprotection from ultraviolet light, eye health, skin health, anti-inflammatory, heart health, health cellular level, anti-cancer, detoxification and improve liver function, immune response, preventing the decline of the central nervous system, and antioxidants (Johnson and An, 1991). While luteine in the retina of the eye and zeaxanthine help protecting the eyes from damage by filtering out blue light, especially in infants and children. According to Bone *et al.* (1992), luteine and zeaxanthine have potential to absorb blue light up to 20-90 percent.

16S rDNA Polymerase Chain Reaction

DNA amplification using 16S rDNA PCR showed positive results with the presence of bacterial DNA isolate TH8 with the appropriate base length of approximately 1500 bp.

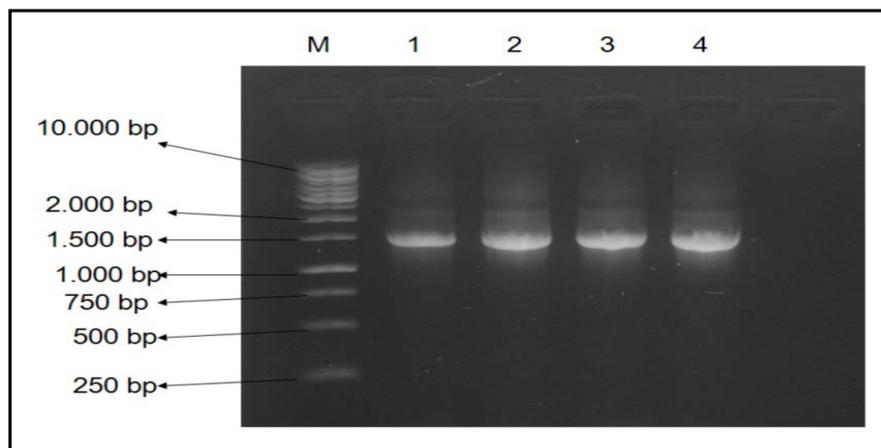


Fig 7. Electrophoresis of single band of 16S rDNA PCR, M = Marker, 2 = TH8

DNA sequences obtained from partial sequencing of 16S rDNA of bacterial isolate follows:

TH8

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TGTGGCTCGTGCCTAATAATGCCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCG
GCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGG
GGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATCATAAAAAGGTGGCTTTTAGCTACCC
CTTACAGATGGACCCCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATG
CGTAGCCGACCTGAAAGGGTGATCGGCCACCCTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCGCATGGACGAAAAGTCTGACGGAACAACGCCGCGTGAGTGA
TGAAGGTTTTTCGGATCGTAAACTCTGTTGTAGGAAGAACAAGTCCGTTTTCGAATAGGGCGGTACC
TTGACGGTACCTAACCAGAAGCCACGGCTAATTCGTGCCACCGCCGCGGA
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The identification of bacterial isolate TH8 with BLAST system can be seen in Table 3 below:

Table 3. Molecular Identification of isolate TH8

No	Strain	Length (bp)	Closest Relative	Homology	Accession
1	TH8	495	<i>Bacillus licheniformis</i> strain CICC	98%	GQ375232.1

Molecular identification results showed that isolate TH8 has the highest percentage of similarity with *Bacillus licheniformis* strain CICC ribosomal RNA gene with a 98% level value. Phylogenetic tree shown in **Fig. 8.** shows the phylogenetic affiliation of bacterial isolate with other microorganisms.

From the results of molecular identification by 16S rDNA, it was shown bacterium TH8 was closely related to *Bacillus licheniformis* with 98% homology value. According to Hagström *et al.*, (2000), isolate which has 16S rDNA sequence similarity similar more than 97 % can represent same species. Therefore we can conclude that isolate bacterial TH8 is *Bacillus licheniformis* species.

Bacillus licheniformis is a group of the genus *Bacillus* belonging to the class of spore-forming rods and cocci. The characteristics of these bacteria are rod-shaped cell length, cell size from 1.5 to 3 milimikron and width from 0.6 to 0.8, this bacterium is a gram-positive bacteria, moving with the flagellate and forms spores. Spores of *Bacillus licheniformis* highly heat resistant. Forms spores ovale or cylinder with a central position. The size of the bacterial wall fibers is proportional to the size of the bacterial cell itself. Composition of cell walls consist of teikoad acid, polysaccharides and poliglican (Herzberg *et al.*, 2004).

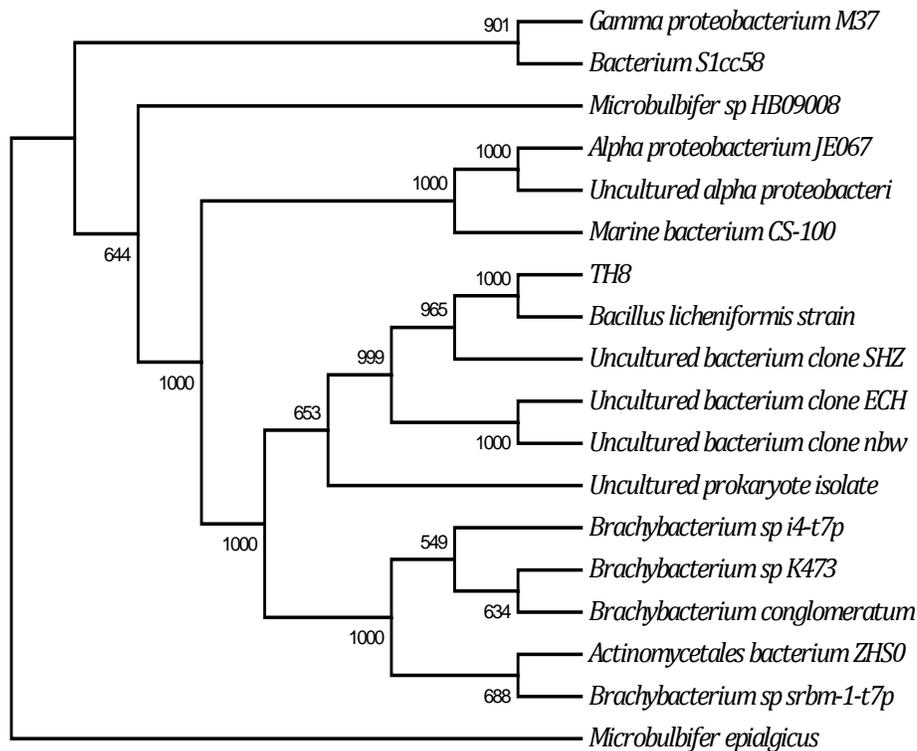


Fig. 8 Phylogenetic tree of TH8 isolate isolated from *Thalassia hemprichii*.

Several species of *Bacillus licheniformis* previously reported have been found in soil and bird feathers, especially birds that tend to stay on the ground rather than air, (sparrow) and water (ducks). These bacteria can cause food poisoning, but also produce protease enzyme used in detergents (Herzberg *et al.*, 2004). Antibiotic-producing *Bacillus licheniformis* is basitrasin types who also produced by *Bacillus subtilis* (Mulati, 1992). Until recently no information on the *Bacillus licheniformis* isolated from sea grass and plants containing carotenoid pigments.

CONCLUSION

Bacterial symbiont, *B. licheniformis* has been confirmed to produce carotenoid pigment content Diadinoxanthine class (Cis, Trans, and Isomers chain). This would open further research avenue about the possible role of bacterial symbionts of sea grasses as the sustainable source of marine natural pigments.

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