Exploration, Isolation, and Identification of Carotenoid from Bacterial Symbiont of Sponge *Callyspongia vaginalis*

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Abstract

During the past two decades research on marine bacteria has highlighted the tremendous potential of symbiotic-microorganisms as a source of bioactive secondary. One of the potential of the bacterial symbionts is producing a natural pigment, and these organisms can be used as a sustainable source of natural pigments. Carotenoid is one of the most important pigments that has important roles in physiological and molecular processes of microorganisms, as well as for human health. The objective of this study is to analyze carotenoid pigments from marine bacterial symbionts from sponge and to identify bacterial symbionts that produce carotenoid pigments. Pigment analysis was performed by a UV-VIS spectrophotometer and High Performance Liquid Chromatography (HPLC). Molecular bacterial identification was performed based on 16S rDNA sequence. The isolation of bacterial symbionts from C. vaginalison Zobell 2216E medium resulted in one bacterium, CB-SP5, positively synthesized carotenoids. By reverse phase HPLC analysis, the carotenoid pigments in the bacterial symbionts were identified as diadinoxanthin, fucoxanthin, neoxanthin, dinoxanthin, and diadinochrome. CB-SP5 shared the highest level of 16S rDNA gene sequence similarity with Psychrobacter celer(99%).

Keywords : carotenoid, sponge, bacterial symbiont, 16S rDNA.

Introduction

Sponges contribute significantly to the total fauna of sessile marine organisms worldwide and dominate the benthic community of some Caribbean and other tropical waters. They are filter-feeding animals because all adult sponges are sessile and cannot move around benthic surface (Hentschel *et al.,* 2002). Various organisms have been found in sponges, including a diverse range of green algae, heterotrophic bacteria, cyanobacteria, archea, cryptophytes, red algae, dinoflagellates and diatoms. One host sponge can possess diverse symbionts. Large numbers of bacteria are also known to be harboured within the extracellular mesophyl matrix of many sponges, living in symbiosis with their host (Imhoff and StÖhr, 2003), or production of secondary metabolites (Unson*et al.,* 1994).

Many of the heterotrophic bacteria that synthesize carotenoids have been isolated from coastal and oceanic waters (Du *et al.* 2006; Satfsnes *et al*., 2006). Some of these bacteria may serve beneficial purposes as the sources of secondary metabolites including marine natural products, such as natural pigments (Radjasa *et al.*, 2003). The accumulating evidences indicate that many bioactive compounds from marine invertebrates are indeed produced by bacterial symbionts (Radjasa *et al.,* 2009). Through the years, pigments have been used as a taxonomic tool for the identification and classification of algae, fungi, and bacteria. The most widely distributed pigments are the carotenoids (Cardona *et al.,* 2010). That also play an important role in bacteria, such as in preventing photo-damage during photosynthetic processes, and conferring resistance to oxidative damage due to the production of oxygen (Harashima, 1989; Takaichi *et al.,*1990).

Carotenoids are yellow to red coloured pigments which originate from terpenoid biosynthetic pathway, and are synthesized by plants, algae and by some fungi and bacteria. They are involved in the photosynthesis as accessory pigments, functioning as antioxidants, light protection pigments and membrane stabilizer (Abdelnasser, 2008). The most important biological function of carotenoids is as antioxidants owing to their potential to inactive singlet oxygen and to quench carboxyl radicals (Di Mascio *et al*., 1989; Burton and Ingold, 1984; Britton, 1995).

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Bacteria isolated from living surfaces, in particular from sponge are a promising source of natural products. Symbiotic marine bacteria with this sponge also have the potential to produce natural pigments (Wusqy *et al.*, 2014). Carotenoid pigment is very feasible to be exploredue to the excellence and diversity of marine microorganisms. In this work, we reported analysis of carotenoid pigments of marine bacterial symbiont from sponge and molecular identification of bacterial symbiont that produce carotenoid pigments.

Materials and Methods

Sampling and isolation of bacterial symbionts

Samples of sponge *Callyspongia vaginalis*, were taken from Cemara Besar Island, Karimunjawa at depth of 5-10 meters manually by using a cutter, which were then put into sterile plastic bag and were stored temporarily in a cool box. The samples were rinsed 3 times with sterile sea water to clean the bacteria that temporarily attached to the surface waters. The tissues were then rinsed with sterile seawater and cut with a sterile knife. The resultant tissues were serially diluted, spread on full strength ZoBell 2216E marine agar medium and incubated at room temperature for ± 72 h (Burgess *et al., 2*003). Petri dish was then incubated at 30°C for three days. On the basis of morphological features, the colonies were randomly picked and purified by making streak plates (Madigan *et al.,*2000).

Extraction of bacterial pigments

Appearance of yellow pigment from the bacterium isolate was started after 72 hours of incubation in the culture medium. Bacterial symbiont was cultured on Zobell agar medium. A total of two grams of pellets were extracted using cold acetone-methanol 35 :15 ml (7:3 v/v) (Cohen-Bazire *et al*., 1957; Kuki *et al.,* 1994), with the aid of a sonicator (Britton, 1995). 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 reversed phase column AB with, C18, having a diameter of 4 mm x 25 mm and a mobile phase of methanol: acetone (Nugraheni *et al*., 2010). Detection of pigment was performed at a wavelength of 190-800 nm with a flow rate 1 ml.min-1, pressure 1000 psi (Maeda *et al.,* 2005).

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, was allowed for an overnight temperature of 4°C. The mixture was centrifuged at 12000 RPM for 10 minutes then1 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 final concentration of the DNA was measured using Eppendorf tube, and then stored at - 20°C (Walsh *et al.,* 2013).

16S rDNA Polymerase Chain Reaction

The universal primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and primer 1492R (5'-TACGGYTACCTTGTACGACTT-3') were used to amplify 16S-rDNA gene (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 2 min), annealing (55°C for 1 min), and extension (72°C for 2 min). Series of denaturation, annealing and extension were repeated until 45 cycles. Electrophoresis was done on 2% agarose. Sequencing was done according to Radjasa *et al.* (2009). Homology search and DNA data bank by BLAST (Altschul *et al.,* 1997).

DNA Sequencing Accession Number

The complete 16S rDNA gene of bacterial isolate CB-SP5 have been determined and deposited in DDBJ. The GenBank accession number is LC214826.

Results and Discussion

Sampling and isolation of bacterial symbionts.

Microbial pigments are a promising alternative to other colour additives extracted from vegetables or animals because they are considered as natural and show high productivity (Rashid *et al.,* 2014). The main and most plentiful pigment group in marine pigmented bacteria is carotenoid which usually appears orange, yellow or red colour (Marit *et al.,* 2010).

In this study, we have successfully isolated a bacterial symbiont of sponge *Callyspongia vaginalis*. Marine sponges are rich sources of natural compounds, which exhibit wide variety of biological activity (Ibrahim *et al.,* 2014). *C. vaginalis* is a solitary species, play an important role in the transfer of energy between the benthic and pelagic environments on coral reefs (Duckworth *et al.,* 2006; Cruickshank, 2016). It is also functionally important for water-column productivity, utilized in a saltwater aquarium to aid in the filtration of suspended food particles (Walters *et al.,* 2005; Cruickshank *et al*, 2016).

Isolate CB-SP5 produce pigment which was expected as source of carotenoids. The bacterial gave a greenish yellow colour (Figure 1).

The carotenoids were extracted and identified based on their chemical, chromatographic and spectroscopic spectral (UV-Vis and mass spectrometry). Analysis by spectrophotometer is the step to identify the presence of pigment in bacterial symbiont of *C. vaginalis.* Absorption spectra UV spectrophotometer are shown in Figure 2.

Absorption spectra pigment extract can be identified as the major pigment in bacterium is carotenoid. Most carotenoids absorbed maximally at three wavelength (Britton, 1995). The wavelength of maximum absorption and the shape of spectrum are characteristic of carotenoid chromophore (Sandmann, 2008).

Figure 3 shows an elution profile on HPLC system that corresponds to the identification for each chromatographic. HPLC has made it possible to simultaneously determine the concentrations of a wide range of carotenoids and chlorophylls and their degradation products (Bidigare *et al.,*2005). Peak 1, 2, and 3 were identified as diadinoxanthin, fucoxanthin, dinoxanthin, respectively. These three peaks (Figure 4) have a maximum absorption paterns that almost the same. The differences of the three peaks are only in stereoisomer which is a compound that has the same molecular formula. Peak 4, which was the major carotenoid, was assigned as neoxanthin. Peak 5 was a minor diadinochrome. There are several another peaks that shown on HPLC chromatogram, were not identified.

HPLC analysis equipped with a PDA detector (Photo Diode Array), excess PDA detector is able to analyze the multi-wavelength and can be see absorption spectra of each peak. Pattern spectra are presented in Figure 4.

On HPLC chromatogram of pigment extract in bacterial symbiont of *C. vaginalis* showed there are only 5 of specific and main pigments based on typical peak and peak area at retention time of 9, 9.4, 13.8, 24, and 43.2 minutes. These peaks identification were based on the absorption maximum of each pigment compared with literature (Table 1). Figure 5 shows the corresponding chemical structures. Results of spectrophotometer with methanol acetone reagents show that the spectral pattern formed have peak absorption at a range of wavelength of 300-600 nm (Gross, 1991).

Diadinoxantines are compounds with chemical structure $C_{40}H_{54}O_3$, pigment is included in xanthophyl group 1 that normally found in diatoms and dinoflagelata. Diadinoxanthin included in the xanthophyl group because it has oxygen in ring ionon. Therefore it can be conclude that the class diadinoxanthin found as carotenoids of the CB-SP5

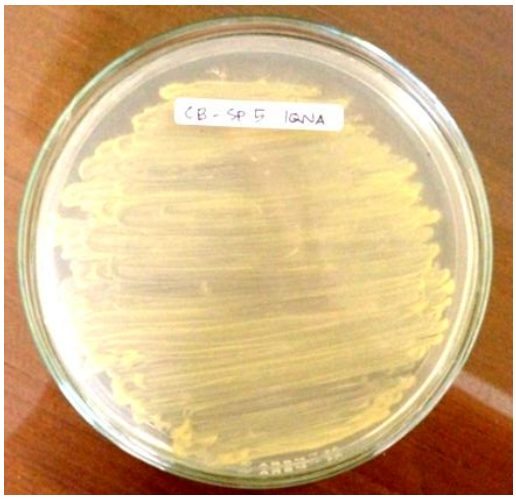

Figure 1. Pigment producing bacterium isolated from sponge sample

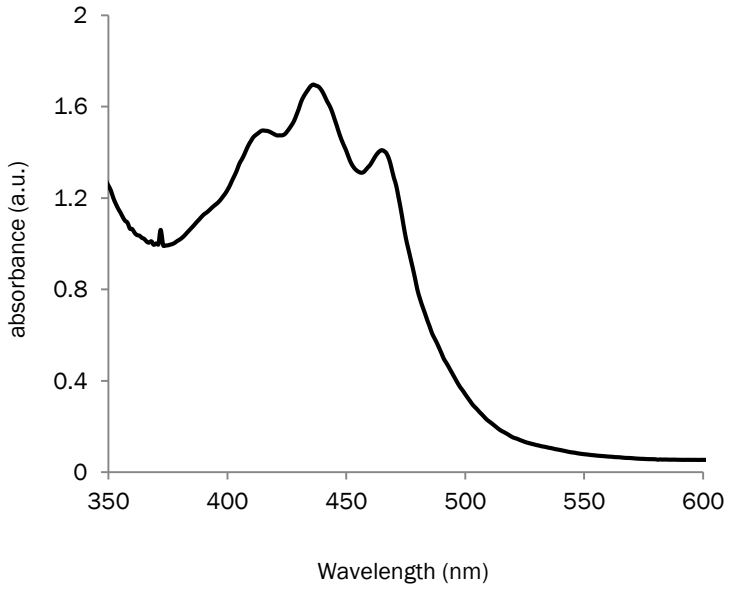

Figure 2. Absorption spectra pigment extract from bacterial symbiont of *Callyspongia vaginalis*

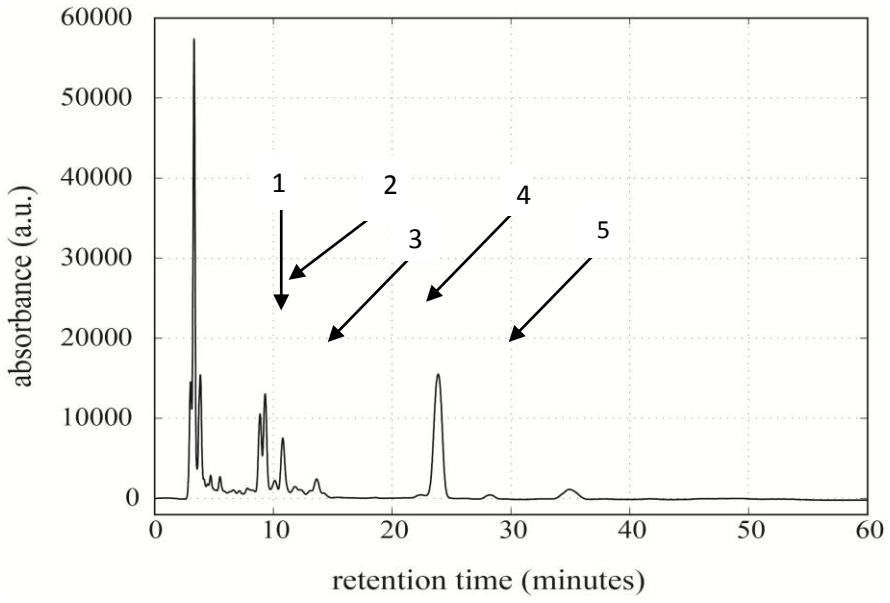

Figure 3. HPLC chromatogram of separation pigment from bacterial symbiont of *C. vaginalis*

isolate is a carotenoid of xantophyl class. Dinoxanthin, fucoxanthin, neoxanthin, are also type of xanthohyll. Diadinochrome is rearrangement product of diadinoxanthin, which found in dinoflagellate.

Many marine sponges are brilliantly colored due to the presence of carotenoids. The characteristic carotenoids in sponges are aryl carotenoids such as isorenieratene, renieratene, and renierapurpurin (Matsuno, 2001). More than twenty aryl carotenoids have been reported in sponges. Except for sea sponges, aryl carotenoids are found only in green sulfur bacteria. Therefore, aryl carotenoids in sponges are assumed to originate from symbiotic bacteria (Matsuno, 2001). Novel carotenoid sulfates having an acetylenic group, termed bastaxanthins, were isolated from the sea sponge *Ianthellabasta* (Britton *et al.,* 2004). Recently, a new acetylenic carotenoid was isolated from the marine sponge *Prianososiros* (Rogers *et al.,* 2005). However, in the present study shows that

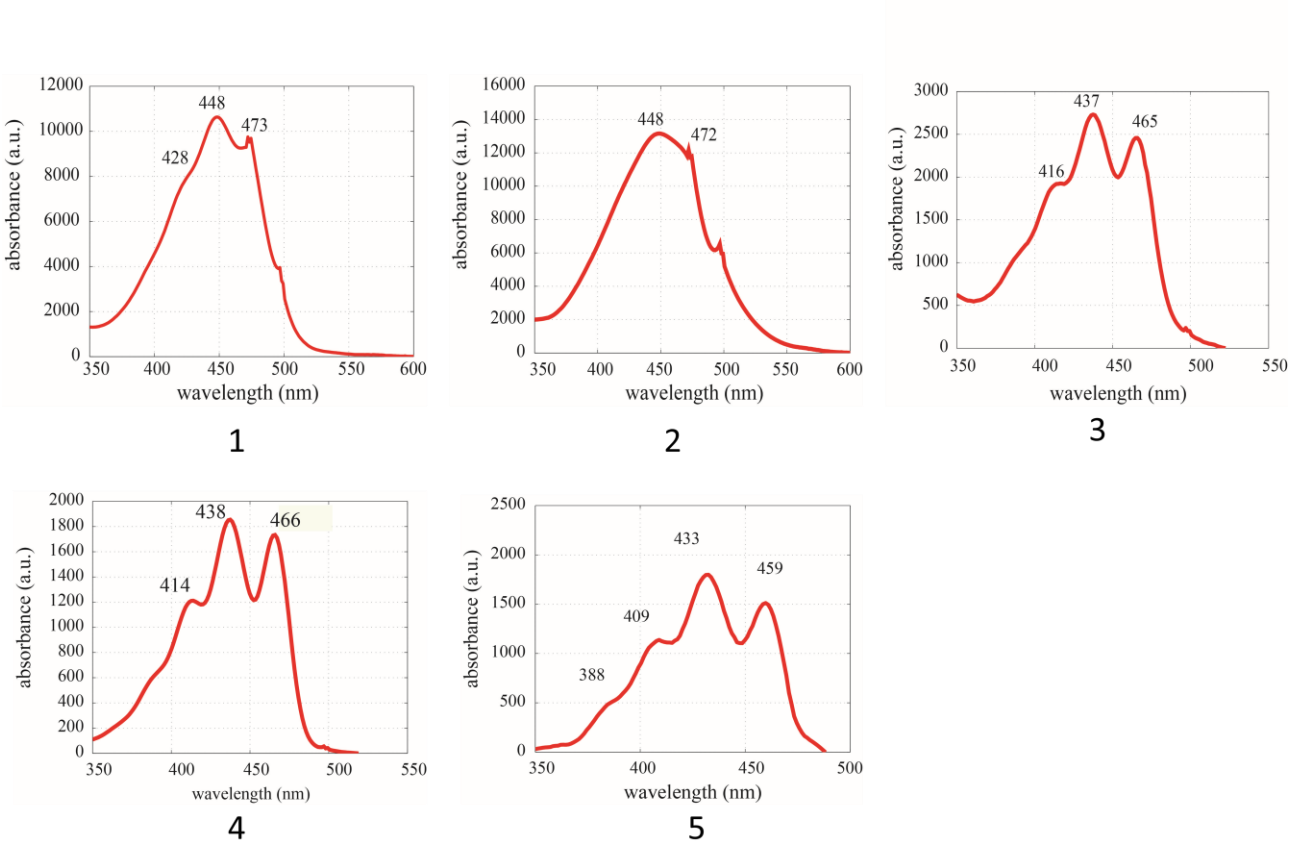

Figure 4. Absorption spectra of typical and main pigments diadinoxanthin (1), fucoxanthin (2), dinoxanthin(3), neoxanthin(4), diadinochrome (5)

Table 1. List of characterization of carotenoid pigments in CBSP5.

Peak	Retention time (min)	Absorption maximum (nm)	
		Result	Jeffrey, 1997
1	9.0	428, 448, 473	425, 448, 478
2	9.4	448, 472	446, 468
3	13.8	416, 437, 465	416.7, 440.5, 469.9
4	24	414, 437, 465	415.1, 438.5, 467.1
5	43.2	409, 432, 459	410, 430.7, 457.9

bacterial symbiont from sponge *C. vaginalis* did not produce the aryl carotenoids.

The present study probably indicates that pigment production is influenced by physical factors such as temperature and pH of the culture medium. There should be many other factors, affecting pigmentation by the bacterium such as source and concentration of nutrient component. The bacteria growing on the surface of sponges live in a highly competitive environment in which access to space and nutrients are limited (Ibhrahim *et al.,* 2014; Slattery *et al.,* 2001). In addition, surfaces of many marine invertebrates providing a nutrient rich habitat for heterotrophic bacteria that leading to the formation of biofilm-forming microbial communities. It has been suggested that natural products from marine invertebrates have striking similarities to metabolites of their associated microorganisms.

Amplification of the 16S rDNA was done by PCR with positive amplification of approximately 1500bp (Figure 6). Molecular identification, by two directions sequencing of the PCR product, showed that isolate CB-SP5 has the highest percentage of similarly with *Psychrobacter celer* strain with a 99% level value (Table 2). 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 CB-SP5 is *Psychrobacter celer* species. Figure 7 shows the phylogenetic affiliation of bacterial isolate with other microorganisms.

The genus *Psychrobacter* was created by Juni and Heym (1986). This organism grew optimally at 25-30°C, occurs at pH 5-0, and in the presence of 2-3 % (w/v) NaCl, which was isolated from the South Sea in Korea (Jung-Hoon *et al*., 2005). The

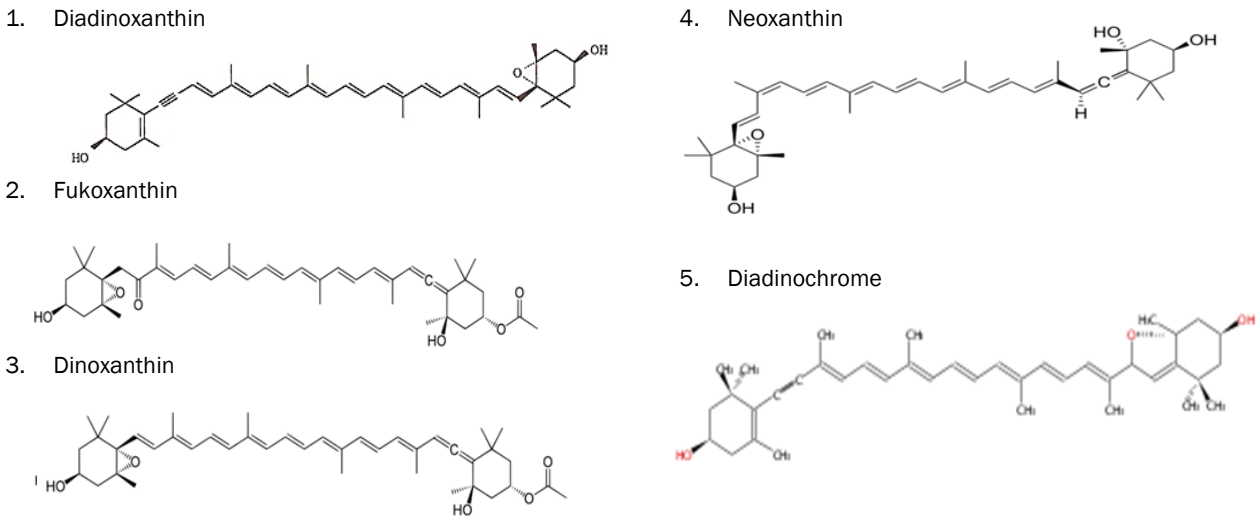

Figure 5. Structure of carotenoids produced by bacterial symbiont of *C. vaginalis* (Jeffrey, 1997)

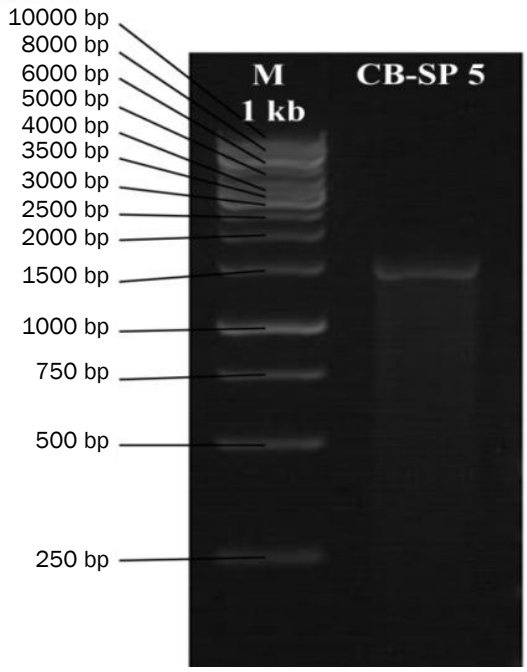

Figure 6. PCR Amplification of 16S rDNA fragment. M : Marker; CBSP5: sample; bp : base pair

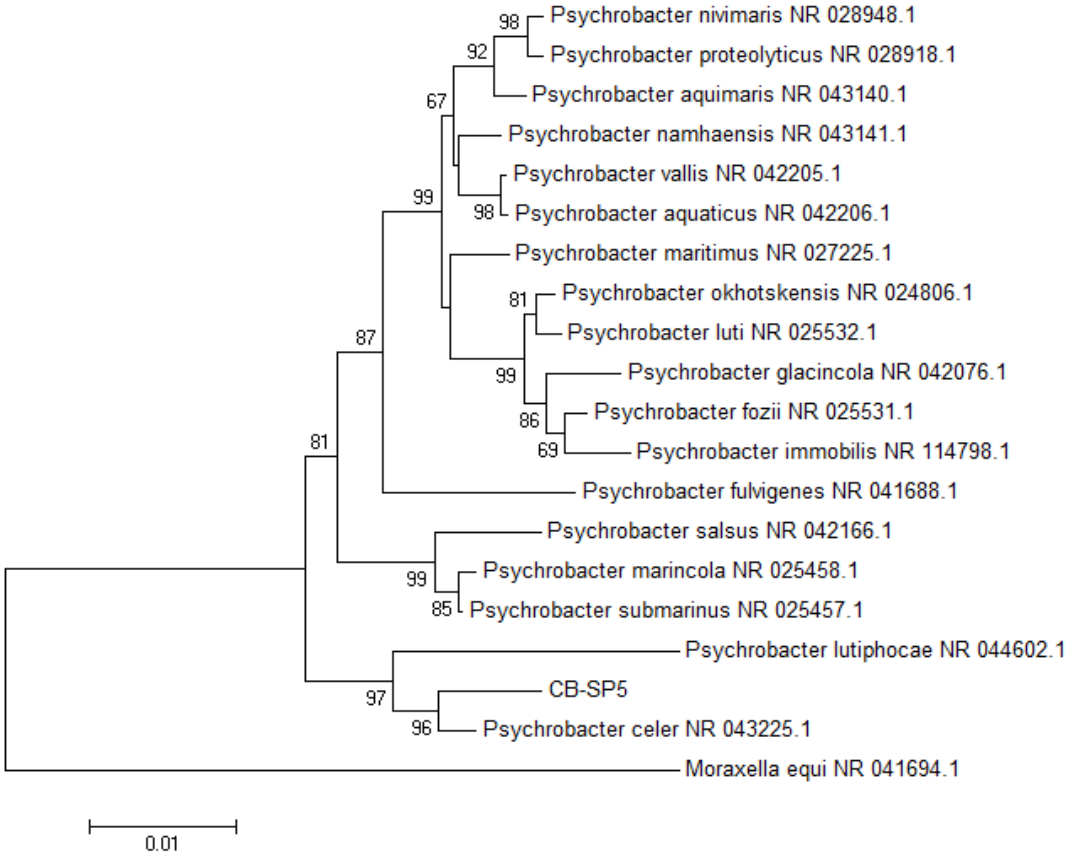

Figure 7. Phylogenetic tree based on 16S rDNA gene sequence strain CB-SP5 and representative members of related species of the genus *Psychrobacter.*

Table 2. Molecular identification of bacterial symbiont

Code	Length	Closest Relative	Homology
CB-SP5	1500bp	*Psychrobacterceler*	99%

characteristics of this bacterium are Gram-negative,nonmotile bacterium, nonspore-forming, slightly halophilic bacterial strain (Jung-Hoon *et al*., 2005). Colonies are circular, smooth, glistening, raised, cream-colored. According to Juni and Heym (1986) that bacteria genus *Psychrobacter* are non-pigmented, and some species of this genus produce the black-brown pigment. However, in this study shows the result that this bacterium *Psychrobacter celer* CB-SP5 can produce carotenoid pigment. It is an interesting phenomenon that needs further research regarding carotenoids that are produced by the isolated bacterium symbiont of sponges.

Surveying and identifying microbial symbionts present in host invertebrates are fundamentally important in cases in which biosynthesis of a natural product. We have just begun identified the pigments as a natural products from the bacterial symbiont of *Callyspongia vaginalis* sponge.

Conclusion

Strain bacteria CB-SP5 which isolated from sponge *Callispongia vaginalis* was identified that have a homology of 98% with *Psychrobacter celer* NR 043225.1. By HPLC analysis, *Psychrobacter celer* CB-SP5 bacteria isolate contains diadinoxanthin, fucoxanthin, dinoxanthin, neoxanthin, and diadinochrome. It was suggests that the bacterial symbionts of *C. vaginalis* have potency as a crotenoid source. However, the study of determining the source of interesting and pigmentation metabolites from bacterial symbiont of sponge has rarely been demonstrated. Thus further research on the bacterial symbiont pigmentation is needed.

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