Identification of Carotenoids in *Halimeda macroloba* Reef Associated Bacteria

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Abstract

Several carotenoid pigments which are produced by sea microorganisms are shown to function as antimicrobe compounds and potential antioxidants. Coral reefs with high levels of diverse biota facilitate Halimeda as a primary component and producer to have associated bacteria that produce relatively unknown metabolic compounds. This research attempts to isolate bacteria that are associated with Halimeda macroloba, identify it, as well as analyze the pigment content produced. A yellow-orange bacteria colony is successfully isolated and given the temporary name MK_HM. This bacteria is in the shape of circular (oval) rods and are gram-variable bacteria. Based on the sequencing analytical results, Blast homology, as well as a phylogenetic analysis, it shows that the bacteria have a relationship with Exiguobacterium aestuarii TF-16 at 94%, so that it can be strongly predicted that the MK_HM strain is a new species. This pigment analysis, which is conducted on the Exiguobacterium sp. MK_HM bacteria extract, reveals that this strain produces carotenoid pigments of diadinochrome, semi-α-carotenone, dinoxanthin, and P457.

Keywords: *Exiguobacterium, carotenoid, diadinochrome, semi-α-carotenone, dinoxanthin, and P457.*

Introduction

Carotenoids are yellow, orange, and red pigments that are found in several animals (Guerin *et al.*, 2003), plants, and microorganisms (Britton *et al.*, 1995a). In the human body, carotenoids play an important role for the health, including as a vitamin A precursor, anti-cancer, antioxidant, to manage night blindness, cataracts, and macular degeneration, as well as xeroftalmia, or eritropoietik protoporfiria therapy (Limantara, 2008). Several carotenoids that are produced by sea microorganisms function effectively as antimicrobes and potential antioxidants (Balraj *et al.*, 2014; Mani *et al.*, 2015). Research developments which show the important role of pigments in health and beauty fields make the sea, which is the largest habitat on earth (Fenical and Jensen, 1993), as an important natural product source. Its natural characteristics, being year-round, as well as having high productivity make carotenoids produced by microorganisms have great potential to be additive coloring replacements or bioactive compounds that are extracted from plants or animals (Arulselvi *et al.*, 2014), so that they avoid extinction (Li, 2009). There are about 38 carotenoid producing bacteria species that are grouped to become 7 classes, which are Flavobacteria, Sphingobacteria, α-Proteobacteria, ɣ-Proteobacteria, Deinococci, Actinobacteria, and Bacilli. The carotenoids which are produced by these bacteria are zeaxanthin, dihydroxyastaxanthin, astaxanthin, canthaxanthin, and several other kinds of carotenoids that have not yet been identified that are produced by bacteria that are classified as *Deinococcus*, *Exiguobacterium*, and *Flectobacillus* (Asker *et al.*, 2012).

Coral reefs have a high diversity of biota, including their microorganisms, so that it is a highly productive ecosystem. A mucus layer that is rich in polysaccharides on shell surfaces are a good media for bacteria growth, so that a bacteria community is formed on this layer (Rheinheimer, 1991). Bacteria which lives on other organisms usually produce the same bioactive compounds as their hosts (Perez-Matos *et al.*, 2007; Radjasa *et al.*, 2007; and Khoeri, 2011). For instance, *Erythrobacter vulgaris* bacteria, which is associated with *Caulerpa racemose* seaweed, have metabolic compound similarities; they both produce chlorophyll, xanthophyll, and fucoxanthin (Khoeri, 2011). Sponges are a source of medicinal raw materials from the sea; however, excessive exploitation will endanger the sponge population. Related with that, research on symbiont microorganisms from sponges to retrieve metabolite compounds produced by sponges was conducted by (Li, 2009; Wusqy *et al.*, 2014).

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Macroalgae, especially *Halimeda,* is a primary component (Lapointe and Thacker, 2002) and primary producer coral reefs (Rees *et al*., 2007). This conveys that *Halimeda* is a superior competitor (Vroom and Smith, 2003; van Tussenbroek and van Dijk, 2007; El-Manawy *et al.*, 2008; Sinutok *et al.*, 2008). Study results reveal that *Halimeda* extract is effective in eliminating several pathogen proteins (Hendri *et al.*, 2015). The high level of biota diversity in coral reefs (Rheinheimer, 1991), facilitates *Halimeda* to have associated bacteria that produce certain metabolic compounds that have not really been exposed. The lack of information about bacteria associated with *Halimeda* is an opportunity for new research to explore this possibility. Therefore, this research attempts to isolate bacteria from *Halimeda macroloba* and identify it using a molecular marker (16S rDNA). A pigment analysis is also done from this bacteria extract to discover the kinds of pigments produced.

Materials and Methods

Sample preparations

The *Halimeda macroloba* seaweed is taken from Menjangan Kecil Island, Karimunjawa Archipelago, Jepara, Central Java, Indonesia, which geographically is located at 110.9^0 E. and 5.6^o S. The bacteria isolate is cultivated in a Zobell 2216E medium, consisting of bactopepton 5 gr, yeast extract 1 gr, and sterile sea water, until it reaches a final volume of 1 liter (added with 15 gr of bactoagar for agar medium) (Radjasa *et al.*, 2007). The material needed for the gram immersion is a pure cultivation of MK_HM bacteria isolate. The solution needed is Hucker's crystal violet (C.V.) (Gram A) coloring solution, mordan lugol's iodine (Gram B) solution, acetone alcohol (Gram C) solution, and safranin color (Gram D) solution. DNA extraction is done by using a 20% mixture of chelex 100 (Walsh *et al.*, 2013). A 16S rDNA partial sequence is amplified with polymerase KAPA enzyme, universal primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), and specific primer Eubacteria 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Isnansetyo and Kamai, 2003). Sequencing 16S rDNA is done by using Big Dye terminator version 3.1 and primer 27F. The sequence is then analyzed by using ABI3130XL, Applied Biosystem. The pigments are extracted by using an acetone solution (Merck, GR for analysis). Pigment detection is conducted by using a ultraviolet-visual (uv-vis) Varian Cary 50 Spectroscope. A pigment analysis is done by using High Performance Liquid Chromatography (HPLC) with methanol eluent (Sasidharan *et al.*, 2013).

Sampling and bacteria isolation

Samples of *Halimeda macroloba* were washed with sterile sea water and put into a cool box. The samples were then extracted by using a mortar and cultivated inside a reaction tube that contains a Zobell 2216E liquid medium for 48-72 hours. After several dilution series are conducted, the culture is spread on a Zobell 2216E agar plate. Then it is incubated at a temperature of 30^{o}C for 48-72 hours (Radjasa *et al.*, 2007). Based on the morphological characteristics, the primary colony, which is yellow-orange, is isolated with a streaking method (Madigan *et al.*, 2000) in a new Zobell 2216E agar plate. Then it is re-incubated at a temperature of 30^{o}C, for 48-72 hours. A series of isolations are done until a pure culture is obtained that originates from 1 colony. This pure cultivation is then multiplied in 25 agar plates, and then it is incubated at the same temperature and time period as before to prepare for pigment extraction.

Identification of MK_HM isolate bacteria

The MK_HM isolate bacteria pure culture is taken aseptically with 1 oz., put on an object glass at the same level, and then dried out and defixated above a bunsen burner. After it has cooled, 2-3 drops of gram A coloring are put on it and let to stand for 1 minute. Next, it is cleaned with flowing water and dried out. After it is dry, the sample has gram B drops put on it, and it is let to stand for 1 minute. Then it is washed with flowing water and dried again. A gram C solution is put on it and let to stand for ± 30 seconds. Then it is washed with flowing water and dried. Last, a gram D solution is put on it and let to stand for 2 minutes. Then it is washed with flowing water and dried. The sample that has been colored is then observed by using a microscope.

DNA isolation is done in the following way: 3 oz. of 48 hour old bacteria culture is placed inside a microtube that contains 100 µL of ddH_2O and added with 1 mL of saponin 0.5%. The bacteria culture is soaked in saponin 0.5% in Phosphate Buffer Saline (PBS) 1X overnight at a temperature of 4^{o}C. The bacteria culture and saponin 0.5% mixture is centrifuged at 12,000 rpm for 10 minutes, and then the supernatant is discarded. Next, 100 µL of ddH_2O + 50 µL of 20% chelex 100 is added to the pellet. First, the chelex mixture is shaken and determined that some crystals of them are found within the sample. After that, the mixture is heated for 10 minutes, and vortexed every 5 minutes. The mixture is centrifuged at 12,000 rpm for 10 minutes. The supernatant that contains the DNA genome is moved to a new Eppendorf tube (Walsh *et al.*,

2013). The purity and quality of the DNA is determined by measuring optical density (OD) at 260 and 280 nm, using NanoDrop-2000 Spectrophotometer.

The PCR mixture formula used is primer 27F: 1.5 µl, primer 1492 R: 1.5 µl, KAPA: 25 µl, ddH_2O: 19 µl, DNA: 3 µl, with a total mixture volume of 50 µl. PCR is done with the following program: early denaturation of 95°C, for 3 minutes. Then it is done for 30 cycles (denaturation 94°C, for 1 minute; annealing 53°C, for 1 minute; extension 72°C, for 1 minute); and then final extension at 72°C, for 7 minutes, and hold at 4°C, ∞. Confirmation of the PCR 16S rDNA product is done with electrophoresis and detected by using a UV transluminator.

DNA sequencing is done by 1st BASE. First, Sequencing PCR is conducted based on a Big Dye terminator version 3.1, with the formula: 2 µl big dye, 2 µl buffer 10X, 4 µl template DNA, 1 µl primer 27F with a concentration of 3.2 pmol, ddH_2O, until the final volume is 10 µl. Amplification is done with an initial denaturation program of 96°C, for 2 minutes; then 25 cycles are done (denaturation 96°C, for 10 seconds; annealing at 50°C, 5 seconds; and an extension of 60°C, for 4 minutes). The DNA is then purified by using a PCR clean-up kit. The sequence is then analyzed by using ABI3130XL, Applied Biosystem. The results are in the form of an electropherogram. The electropherogram data is processed by using a BioEdit (BioEdit Sequence Alignment Editor 7.1.3.0) program to conduct an alignment between DNA sequences.

The sequence data that is obtained from the 16S rDNA sequencing result in the sample is matched with the DNA sequences that is obtained in the gene bank (www.ncbi.nlm.gov). Because it is a bacteria sample, so it has the menu: Search bacterial and fungal rRNA sequences with Targeted Loci BLAST. Then the sequence is compared with a comparison sequences. The comparison sequence used is a DNA 16S sequences from a database in the gene bank (www.ncbi.nlm.gov). Sequence is compared by aligning the sequences using ClustalW in Bioedit software (Hall, 1999). The aligning results are used to form a phylogeny tree using Mega 6 software.

Pigment analysis

Twenty-five agar plates of bacteria that have been previously prepared are carefully scraped. Then they are smoothened using a mortar by adding acetone as a solvent. Extraction is done by being exposed to nitrogen gas to maintain dampness. The extraction results are then strained using filter paper (Whatman, 0.2 µm). The yellow-orange filtrate obtained is confirmed by using a uv-vis spectroscope. Pigment detection is done by using a uv-vis Varian Cary 50 spectroscope in wavelengths of 350-800 nm. A pigment analysis is then done with HPLC with a uv-vis detector. The sample is analyzed by using a C-18 reverse-phase column, with a gradient elution from 80 to 100% methanol, with flowing speed of 1ml/minute. It is run for 50 minutes, and the sample is monitored at wavelengths of 470 nm. The absorption spectrum is recorded by using a photo diode array (PDA) detector (Sasidharan *et al.*, 2013).

Results and Discussion

Bacteria sampling and isolate

From the screening results, many bacteria colonies were obtained. Several among them revealed light yellow, yellow, until yellow-orange morphological characteristics, which are suspected of containing carotenoid pigments. The colonies that were strongly suspected of containing carotenoids were isolated by using new Zobell 2216E agar plates. One of the candidates was successfully isolated, which was a colony that exhibited a yellow-orange color. It was then given the temporary name MK_HM. Next, the MK_HM colony was used as a subject in this research .

Identification of Bacteria

Identification of the MK_HM isolate was first done by observing the colony morphology in a macroscopic and microscopic method. The bacteria colony was in a circular (data not included) shape, curved shape, and yellow-orange color. Observing the bacteria microscopically in the stationary phase revealed that the bacteria had a short rod shape with circular ends, or in an almost coccus shape (rod-oval). This shape possibly made the metamorphosis shape of the bacteria during the growth stage from the rod shape in the early growth stage. Then it shortened so that it tended to take a coccus shape in the exponential and stationary phase like in several kinds of *Exiguobacterium* bacteria, like *Exiguobacterium undae* sp. nov., *Exiguobacterium antarcticum* sp. nov. (Frühling *et al*., 2002), *Exiguobacterium aestuarii* sp. nov., and *Exiguobacterium marinum* sp. nov. (Kim *et al*., 2005). The gram coloring results reveal that these bacteria are gram-variable bacteria (Figure 1) (Kim *et al.*, 2005).

This conclusion is based on references that in general *Exiguobacterium* are gram-positive bacteria (Chaturvedi *et al.*, 2008; Crapart *et al*., 2007; Shatila *et al*., 2013; Mani *et al*., 2015; Zhang

Table 1. BLAST search results of MK_HM bacteria isolate

No	Code	Length	Closest Relative	Homology	Accession
1	MK_HM	1432	*Exiguobacterium aestuarii* strain TF-16	94 %	NR_043005.1

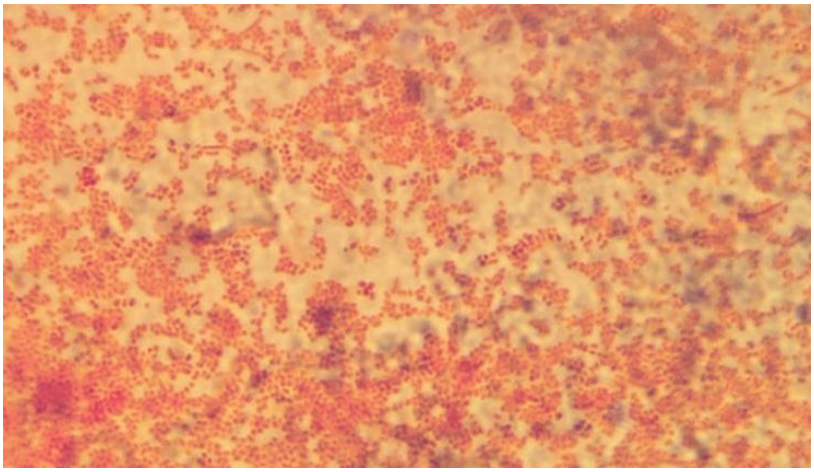

Figure 1. Microscopic observation results of MK_HM isolate bacteria colony. Bacteria is in the form of short rods with both ends circular (rods-circular), gram-variable.

et al., 2015). However, there are several kinds that reveal different results. They become red or a red and purple mixture towards the treatment of gram coloring like in *E. aestuarii* sp. nov. and *E. marinum* sp. nov., so that both of these species are categorized in gram-variable bacteria (Kim *et al*., 2005).

Region 16S rDNA MK_HM isolate bacteria which was 1432 bp. and successfully amplified (Figure 2). By a sequencing analysis, a partial sequence of 16S rRNA MK_HM bacteria isolate was revealed (Figure 3). The sequence was then compared with DNA sequences in the DNA data basis (Altschul *et al*., 1997). The BLAST homology analytical results from the 16S rDNA partial sequence reveals that the MK_HM isolate is closely related to *Exiguobacterium aestuarii* TF-16 with an identity level of 94% (Table 1). With this 94% similarity, the MK_HM isolate can be said to have the same genus (Petti, 2007) with *E. aestuarii* TF-16, which is *Exiguobacterium* genus. The phylogenetic analysis also reveals that the MK_HM isolate has a close relationship with *E. aestuarii* TF-16 (Kim *et al.*, 2005), where it was found that *E. aestuarii* TF-16 is a novel isolate (Kim *et al.*, 2005). Based on this analysis, it can be predicted that the MK_HM isolate is also a novel isolate. The close association with *E. aestuarii* (Kim *et al.*, 2005), strengthens the conclusion that *Exiguobacterium* sp. MK_HM is gram-variable bacteria.

Exiguobacterium is bacteria from the coryneform group. It grows in aerobic or anaerobic environments (Frühling *et al.*, 2002: Yumoto *et al.*, 2004), and is gram positive (Chaturvedi *et al.*, 2008; Crapart *et al*., 2007; Raichand *et al*., 2012; Shatila *et al.*, 2013; Mani *et al.*, 2015; Zhang *et al*., 2015). However, several species are gram variable bacteria like *E. aestuarii* sp. nov. and *E. marinum* sp. nov. (Kim *et al.*, 2005). They have various shapes, as some are rods (Crapart *et al.*, 2007; Keynan *et al.*, 2007; Mani *et al.*, 2015; Yumoto *et al.*, 2004), short

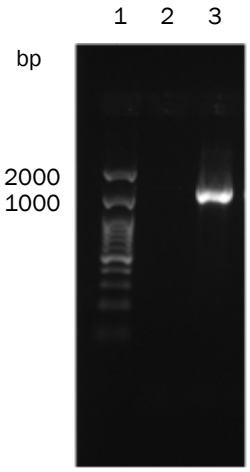

Figure 2. Confirmation of *16*S rDNA MK_HM bacteria isolate. There is an amplification of 16S rDNA MK_HM bacteria isolate with a length of 1432 bp. 1: Maker, 2: Control, 3: MK-HM

rods (Raichand *et al*., 2012), coccus (Shatila *et al*., 2013), and even there are those which experience changes in shape during growth, where the shape of the rods or short rods during the early growth stage then become shorter (Frühling *et al*., 2002), so that they become coccus in the stationary phase (Kim *et al*., 2005). *Exiguobacterium spp*. have a wide habitat, including air (Sasidharan *et al*., 2013; Shatila *et al*., 2013), land (Sasidharan *et al*., 2013), sea (Zhang *et al*., 2015), and even in extreme environments like the snow in Antarctica (Frühling *et al*., 2002), Himalayan glaciers (Chaturvedi and Shivaji, 2006), Siberian ice mountains (Rodrigues *et al*., 2006), deep-sea hydrothermal vent (Crapart *et al*., 2007), hot water sources (Vishnivetskaya *et al.,* 2009), and even high alkali content sources (Cabriaa *et al*., 2014).

With it being spread out all over, it shows that *Exiguobacterium* are bacteria that possess a great ability to survive, including in facing extreme temperature pressures of -5^{o}C (Rodrigues *et al.*, 2006) until 53^{o}C (Zhang *et al.*, 2015), even though every species has different optimum temperature ranges. Several species from *Exiguobacterium* like *E. undae*, *E. antarcticum* (Frühling *et al.*, 2002), *Exiguobacterium oxidotolerans* sp. nov. (Yumoto *et al*., 2004), *Exiguobacterium aquaticum* sp. nov. (Raichand *et al*., 2012), *Exiguobacterium aurantiacum* (Shatila *et al*., 2013, and Sasidharan *et al*., 2013), *Exiguobacterium profundum* (Crapart *et al*., 2007; Sasidharan *et al.*, 2013; Mani, 2015), produce yellow until orange metabolites, which are suspected being carotenoids (Sasidharan *et al.*, 2013, Shatila *et al*., 2013).

Pigment identification

Pigment detection by using a uv-vis Varian Cary 50 spectroscope revealed maximum absorption at wavelengths of 451 nm with 2 shoulders on the left and right, indicating a carotenoid spectrum (Figure 5). A pigment analysis by using HPLC revealed several carotenoid spectrums with varied retention time (Rt.) (Figure 6). Based on the peaks of each spectrum analysis, several peaks can be

GGCGTCCTATAATGCAGTCGAGCGCAGGAGCCGTCTGACCCTTCGGGGGGACGACGGTGGAATGAGCGGCGGACGGGTGAGTA
ACACGTAAAGAACCTGCCCATAGGTCTGGGATAACCACGAGAAATCGGGGCTAATACCGGATGTGTCATCGGACCGCATGGTCCG
CTGATGAAAGGCGCTCCGGCGTCGCCCATGGATGGCTTTGCGGTGCATTAGCTAGTTGGTGGGGTAACGGCCCACCAAGGCGAC
GATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA
TCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAACGATGAAGGCTTTCGGGTCGTAAAGTTCTGTTGTAAGGGAAG
AACAAGTGCCGCAGGCAATGGCGGCACCTTGACGGTACCTTGCGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTCTTAAGTCTGATGTGAAAGCCCCCGGCTC
AACCGGGGAGGGCCATTGGAAACTGGGAGGCTTGAGTATAGGAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGA
GATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGCCTATAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGAAGCTAACGCATTAA
GCACTCCGCCTGGGGAGTACGGTCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGACCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGAAGCAACGCGAAGAACCTTACCAACTCTTGACATCCCCCTGACCGGTACAGAGATGTACCTTCCCCTTTCGGGGGGCAG
GGTTGACAGGTGGGTGCATGGTTTGTCGTCAGCTCGTGGTCGTGAGAATGTTGGGGTTAAGTCCCCGCAACCGAGCGCAACCCTT
TGTTCCTTAGTTTGCCAGGCATTTGGGTTGGGGCACTTCTAAGGGAGACTGCCGGGTGAACAAACCCGGAAGGAAGGGTGGGGG
ATGAACTTCAAATCATTCATGGCCCCTTATGAAGTTGGGCCTACCACCGTGGCTTCAATGGGACGGGTACCAAAGGCCAGCCAAAA
CCCCCGAGGTGGAACCCATTCCCAAAAAAGCCGTTTCTTCAGTTCCGAATTGCAGGGCTGCAACTCCTCCTGCATGGAAATCGGAA
ACCCCCAGAAATCCCCGGGCCAACCATACTGCCGGGTAAAACATTTTCCCGGGTCTGTGGACAACCCGCCCGG

Figure 3. Results of 16S rRNA partial sequence from MK_HM isolate.

Table 2. Dominant peak data

No	Rt[a)]	Area	Height	Area %	λmax			Pigment
1.	19.976	183788	6708	5.4155	414	436	465	Unknown
2.	21.462	102306	3412	3.0146		432	459	Diadinochrome
3.	26.781	607276	20321	17.894	438	464	491	Semi-α-crotenone
4.	27.783	371237	11293	10.9389		441	467	Dinoxanthin
5.	30.302	96474	3868	2.8427	427	457	485	Unknown
6.	31.764	455276	23788	13.4152	438	464	491	Semi-α-carotenone
7.	32.331	153917	9117	4.5353		457		P457
8	33.666	217371	5761	6.4051		442		Unknown

[a)]Rt: Retention time

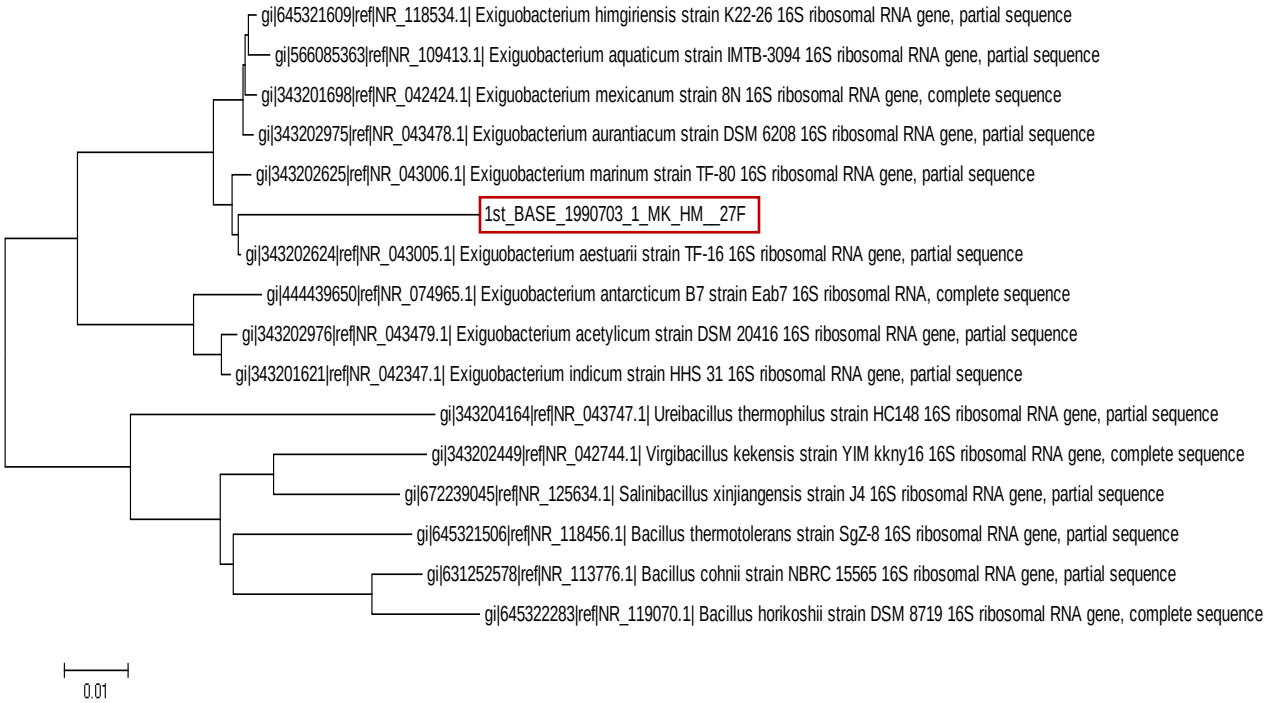

Figure 4. Neighboring-joining phylogenetic tree reveals the position and level of MK_HM bacteria isolate relationship with other microorganisms based on a 16S rRNA sequence. By using a kimura 2 parameter model method and bootstrap 1000 resampling value, a close relationship can be seen with the *Exiguobacterium aestuarii* strain TF-16 bacteria.

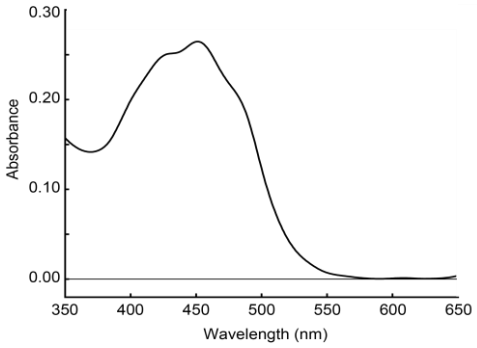

Figure 5. Spectrum of *Exiguobacterium* sp. MK_HM bacteria pigment crude extract by using a uv-vis Varian Cary 50 spectroscope.

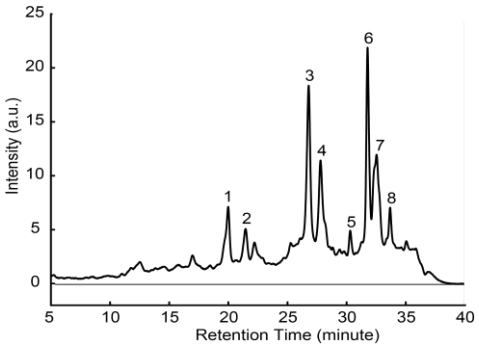

Figure 6. Pigment analysis from *Exiguobacterium* sp. MK_HM bacteria with HPLC. There is a detection with wavelengths of 470 nm.

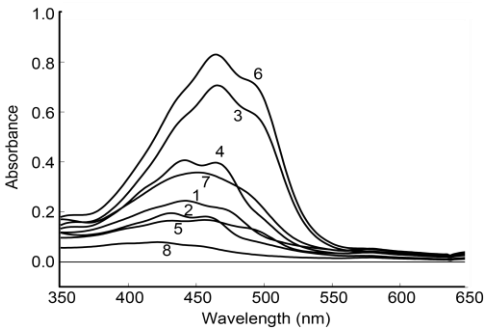

Figure7. Carotenoid spectra pattern from *Exiguobacterium* sp. MK_HM bacteria extract. Peak 2, Diadinochrome; Peak 3, Semi-α-carotenone; Peak 4, Dinoxanthin; Peak 6, Semi-α-carotenone; and Peak 7, P457.

predicted among them, such as Peak 2 is diadinochrome and arises in the 21st minute (Figure 9) (Jeffrey *et al*., 1997). Peak 3 surfaces in the 27th minute (Figure 7), and Peak 6 reaches the 32nd minute (Figure 7). They are both semi-α-carotenone (Britton *et al*., 1995b). Peak 4 is dinoxanthin, which appears in the 28th minute (Jeffrey *et al*., 1997), while Peak 7 is P457, which surfaces in the 32nd minute (Jeffrey *et al*., 1997). Among the five peaks identified, peak 3 and peak 6 (semi-α-carotenone) appear with the highest intensity with a wide area percentage of 17.9% and 13.4%. Then it is followed by dinoxanthin at 11% and P457 at about 4.5%, and the last diadinochrome at 3.0%.

As the semi-α-carotenone appeared 2 times with the highest percentage, indicating that *Exiguobacterium* sp. MK_HM has great potential as a semi-α-carotenone producer. Semi-α-carotenone is a kind of carotenoid that is still rarely found in nature, just in the fruit *Murraya exotica* (orange jasmine) (Yokoyama and Guerrero, 1970), and there is not much research that explains about the characteristics or usage, so that semi-α-carotenone is a new opportunity for research and development of kinds of carotenoids. These research results also show that *Exiguobacterium* sp. MK_HM is a potential dinoxanthin producer. With its antioxidant activity towards reactive oxygen, dinoxanthin (Rodriguez *et al*., 2009) can be made to become an important carotenoid to be researched and developed as a potential antioxidant. Its role as a precursor for other carotenoids like peridinin and P457 (Wakahama *et al*., 2012) also make this dinoxanthin as a crucial target to be studied through a biosynthetic pathway. P457 is also dominantly produced by this kind of bacteria. As is known in the biosynthesis of dinoflagellate, P457 is usually always accompanied by peridinin (Wakahama *et al*., 2012), but in this research it was not identified whether there was a potential for peridinin as a producer from *Exiguobacterium* sp. MK_HM. This is an interesting phenomenon that needs further research regarding carotenoids that are produced by this isolated bacterium. Diadinochrome is a conversion of the diadinoxanthin epoxide group to become furanoid to form epimer 8R and 8S diadinochrome. Diadinochrome, dinoxanthin, and P457 are usually found in species from dinoflagellate (Jeffrey *et al*., 1997). Dinoflagellate is oftentimes symbiotic with coral reefs, so that it makes sense that diadinochrome is also found in *Acropora cervicornis* and *Porites porites* coral extract (Torres-Pérez *et al*., 2012).

Thus, *Exiguobacterium* sp. MK_HM, which is associated with *Halimeda macroloba*, produces the pigments diadinochrome, dinoxanthin, and P457, as well as in dinoflagellate, besides the pigment semi-α-carotenone, which is rarely found naturally, especially in bacteria. According to references, natural semi-α-carotenone has been isolated from the fruit *Murraya exotica* (orange jasmine) (Yokoyama and Guerrero, 1970), and up until now there are no reports about semi-α-carotenone pigment producing bacteria. This study also reveals that *Exiguobacterium* sp. MK_HM is the first semi-α-carotenone producing bacteria. Based on references, it was also found that several carotenoid pigments that are produced by *Exiguobacterium* have not yet been identified (Asker *et al*., 2012). Similar results were also found in this research, in that the kinds of carotenoids produced by *Exiguobacterium* sp. MK_HM are considered as seldom found in other organisms. Regarding the presence or absence of pigment similarities produced by *Halimeda macroloba* with *Exiguobacterium* sp. MK_HM as 2 associated species, further research is still needed, especially about the pigment content of *Halimeda macroloba*.

Conclusion

MK_HM bacteria isolate, which is associated with the *Halimeda macroloba* sampling results in the Menjangan Kecil marine water, Karimunjawa, has a homology of 94% with *Exiguobacterium aestuarii* TF-16, so that it is classified as genus *Exiguobacterium.* Based on the pigment analysis by using HPLC, the *Exiguobacterium* sp. MK_HM bacteria isolate contains diadinochrome, semi-α-carotenone, dinoxanthin, and P457 carotenoid pigments. A further analysis is needed towards this *Exiguobacterium* sp. MK_HM, in order to determine that the predicted species is a new species. Further pigment studies are also needed to find out the advantages of the four pigments identified, so that they can be more useful in solving human problems.

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