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Exploration, Isolation and Quantification of *β*-carotene from Bacterial Symbion of *Acropora* sp.

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In the microbial world, pigments are one of the most conspicuous traits. Marine bacteria associated with *Acropora* sp. collected from Taka Cemara, Karimunjawa Islands were screened for the production of a yellow pigment. The isolation of bacterial symbionts from *Acropora* sp. on Zobell 2216E medium resulted in one bacterium, KJ5, positively synthesized carotenoids. By reverse phase HPLC analysis, one peak of the pigment types was identified as a β-carotene peak which appeared at 60.24 min. Then, sample of the β-carotene was collected and identified according to their spectral characteristics and compared with the published data in different types of solvent. Based on the HPLC analysis, the total β-carotene contents were calculated by converting the broad absorption of β-carotene. Molecular identification of the bacterium KJ5 using 16S rDNA showed that bacterium KJ5 was closely related to *Erythrobacter flavus* with 96% homology value.

Key words: 16S RDNA, *Acropora* sp., β-carotene, *Erythrobacter flavus*, HPLC

Bakteri laut berwarna kuning telah berhasil diisolasi dari karang lunak *Acropora nasuta* yang berasal dari Taka Cemara, Karimunjawa, Jawa Tengah. Dari hasil skrining pada media ZoBell 2216E ditemukan satu isolat KJ5 yang diduga mengandung karotenoid. Identifikasi karoten dengan fase terbalik kromatografi cair kinerja tinggi (KCKT) berhasil mengidentifikasi keberadaan β-karoten yang muncul pada menit ke 60,24. β-karoten murni diperoleh dengan menampung hasil KCKT yang selanjutnya dianalisa dengan UV-Tampak. Spektra diidentifikasi sesuai dengan karakteristiknya dan dibandingkan dengan referensi lain dalam berbagai jenis pelarut. Identifikasi spesies bakteri yang dilakukan menggunakan reaksi berantai polimerase, menunjukkan bahwa isolat bakteri KJ5 memiliki homologi sebesar 96% dengan *Erythrobacter flavus*.

Kata kunci : β-karoten, *Acropora* sp., *Erythrobacter flavus*, HPLC, 16S RDNA

Natural pigments with an annual growth rate of 5-10%, have now comprised 31% of the worldwide colorant market compared to 40% for synthetic colorants (Downham and Collins 2000; Mapari *et al*. 2010). Natural *β*-carotene is an orange-yellow pigment of carotenoid family that is widely used as a food colorant. *β*-carotene is very attractive as natural food colorant due to its antioxidant and pro-vitamin activities which provide additional value to the products (Paz *et al*. 2012). Recently, the price of extracted and purified natural *β*-carotene is much higher than that of synthetic *β*-carotene ($1000 to $2000 kilogram-1 for natural versus $400 to $800 kilogram-1 for synthetic). The price difference reflects that the consumers prefer the natural products to the synthetic *β*-carotene (Caswell and Zilberman 2000).

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Many of the heterotrophic bacteria that synthesize carotenoids have been isolated from coastal and oceanic waters (Du *et al.* 2006). The widespread occurrence of carotenoids in non-phototrophic bacteria suggests that theirpresence is crucial for the viability of these organisms in their natural environment. Due to the absence of photosynthetic apparatus, the importance of the carotenoids in these microorganisms lies mainly in protecting the microbes from photo-oxidative damage and in absorption of visible light (Britton *et al.* 1995). In this work, we reported the identification of a marine bacterium associated with soft coral *Acropora nasuta* and its potential for the productionof *β*-carotene.

MATERIALS AND METHODS

Collection of Samples and Bacterial Isolation. Colonies of soft coral *Acropora nasuta* were collected

from Taka Cemara Karimunjawa waters manually. Upon collection colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA) and put into a cool-box. The tissues were then rinsed with sterile seawater and cut with a sterile knife. The resultant tissues were serially diluted, spread on ½ strength ZoBell 2216E marine agar medium and incubated at room temperature for 48 h. On the basis of morphological features, 9 colonies were randomly picked and purified by making streak plates (Madigan *et al*. 2000).

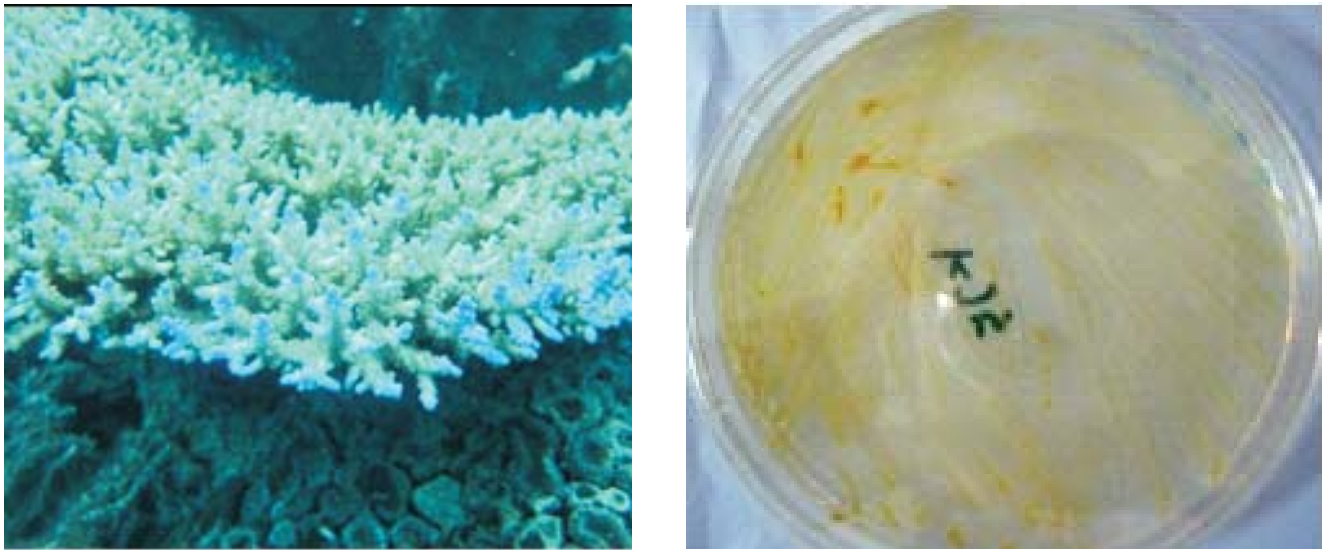
16S rDNA Polymerase Chain Reaction. The universal primers 27F (5'-AGAGTTTGATCMTGGC TCAG-3') and primer 1492R (5'-TACGGYTACCTT GTTACGACTT-3') were used to amplify 16S-rDNA gene (Long and Azam 2001). The temperature cycle of amplification was as follows: initial denaturation at a temperature of 94 °C for 2min, and then successive denaturation (94 °C for 1 min), annealing (55 °C for 1 min), andextension (72 °C for 2 min). Series of denaturation, annealing and extension wererepeated until 45 cycles. Electrophoresis was done on 2% agarose. Sequencing was done according to Radjasa *et al.* (2007). Homology search and DNA data bank by BLAST (Atschul *et al.* 1997).

Extraction of Pigments. Ten plates of bacterial symbiontswhichwere cultured on Zobell agar medium were collected and their pellets moisture was measuredby balancing moisturizer. Then, 10 mL of 100% acetone were added into pellets for extraction (Khalil and Varananis 1996), with the aid of a sonicator (Britton *et al*. 1995).

Identification and Analysis of β-carotene. *β*-carotene was identified and analyzed by using High Performance Liquid Chromatography in reverse phase column AB with ODS, C18, diameter of 4 mm x 25 mm. The method used consists of an elution gradient of methanol, acetone and ammonium acetate solution (1 M), similar to the method used by Hegazi *et al*. (1998). The flow-rate was 1mL min^{-1}, and the gradient protocol lasted approximately 80 min. All these steps were carried out at room temperature. Peak of β-carotene which appeared at 60.24 min was collected and identified according to their spectral characteristics and compared with the published data in different types of solvent.

RESULTS

Sampling and Isolation of Bacterial Symbionts. *Acropora nasuta* samples used in this study were taken from Taka Cemara, Karimunjawa Islands (Fig 1A). One isolate, namely KJ5 (Fig 1B) which was found to produce yellow pigment was expected as source of carotenoids. A total of 200 g wet weight of KJ5 isolate were extracted, resulted in 0.42 g of yellow pigments with 47.10% moisture.



A **B**

Fig 1 Sample of *Acropora nasuta* from Taka Cemara, Karimunjawa, Jepara (A) ; Sample of KJ5 isolate (B).

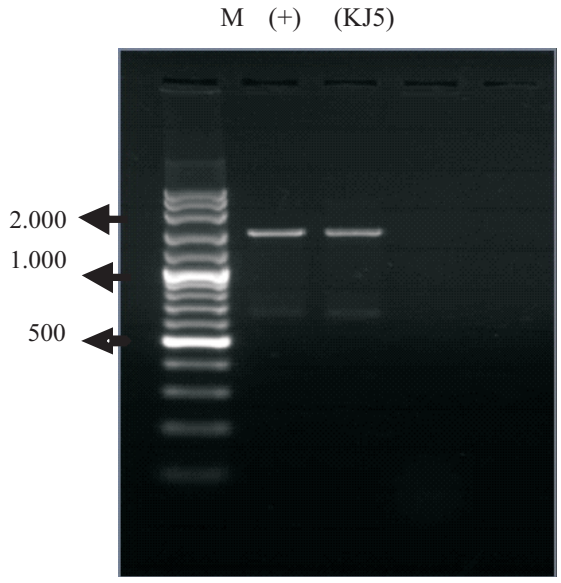


Fig 2 PCR Amplification of 16s rDNA fragment. M: Marker ; (+): positive control; KJ5: sample.

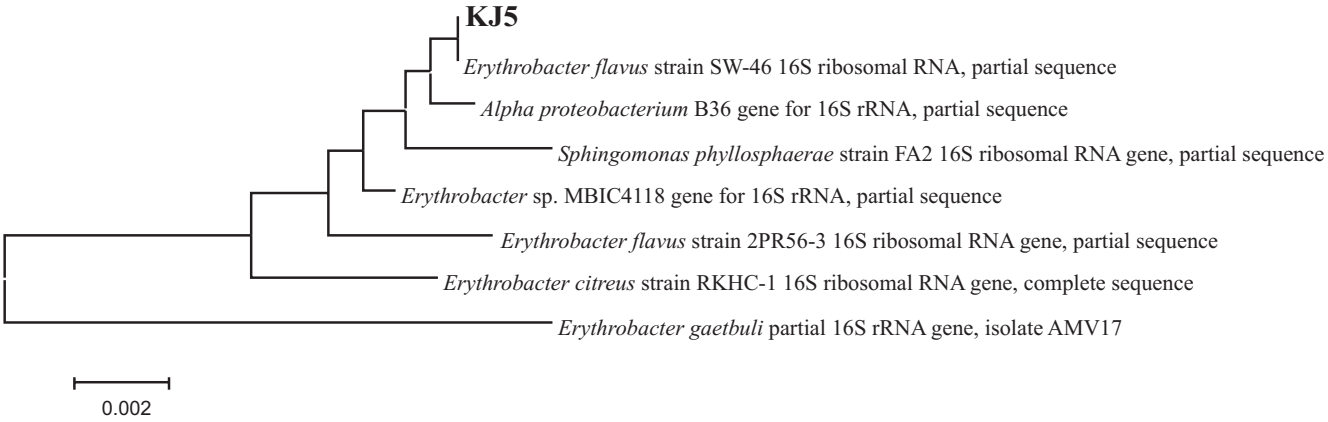


Fig 3 Phylogenetic tree based on 16S rRNA gene sequences of strain KJ5 and representative members of related species of the genus *Erythrobacter*.

Table 1 Molecular Identification of KJ5 isolate

Code	*Length*	*Closest Relative*	*Homology*
KJ5	1440 bp	*Erythrobacter flavus*	96 %

DNA amplification of isolate KJ5 using 16S rDNA PCR showed positive results with the presence of bacterial DNA isolate KJ5 with the appropriate base length of approximately 1500 bp (Fig 2). Phylogenetic tree shown in Fig. 3 shows the phylogenetic affiliation of bacterial isolate with other microorganisms. Molecular identification, by two directions sequencing of the PCR product, showed that isolate KJ5 has the highest percentage of similarity with *Erythrobacter flavus* strain with a 96% level value (Table 1).

From the results of HPLC analysis during 80 min, we found the β-carotene absorption wavelength of 427, 449 and 477 nm at 60.24 min (Fig 8) (Hegazi *et al*. 1998). The spectra of HPLC pigment pattern of KJ5

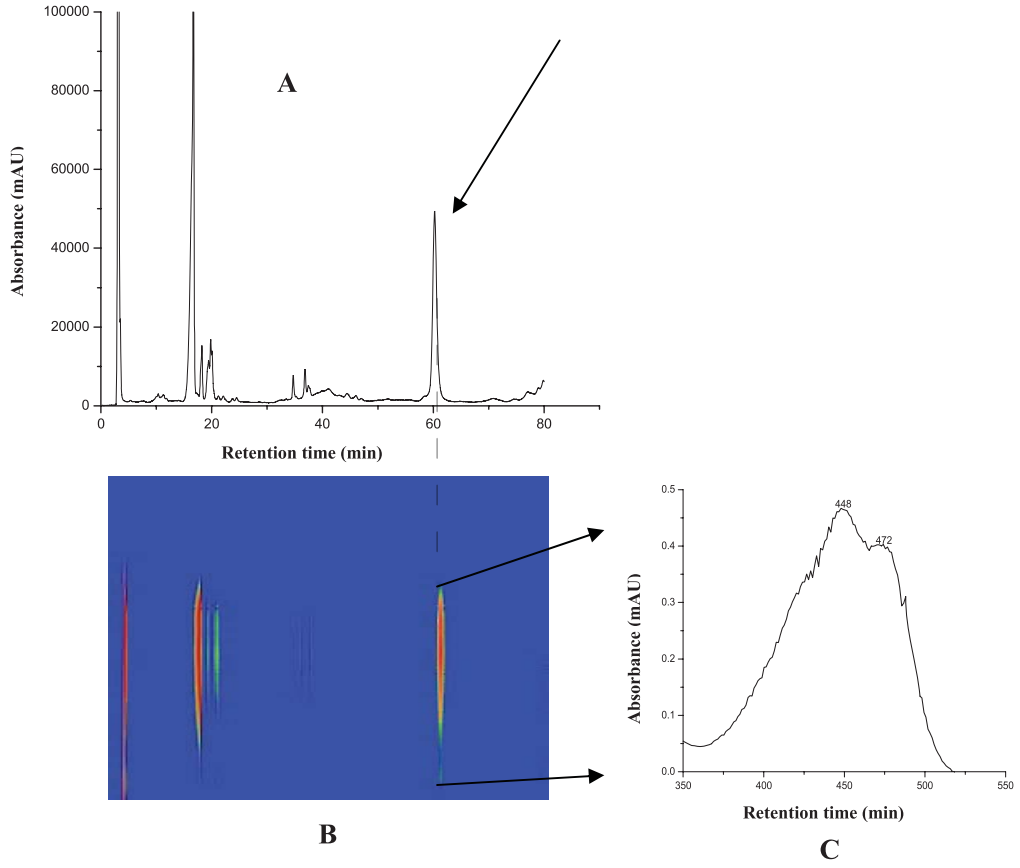


Fig. 4 High-performance liquid chromatogram of acetone extract from *Erb. Flavus* at 450 nm wavelength (A); 2 dimension of extract pigment of *Erb. flavus* (B); Absorption maxima of β-carotene extract at 60,24 min by UV-Vis *spectroscopy* (C).

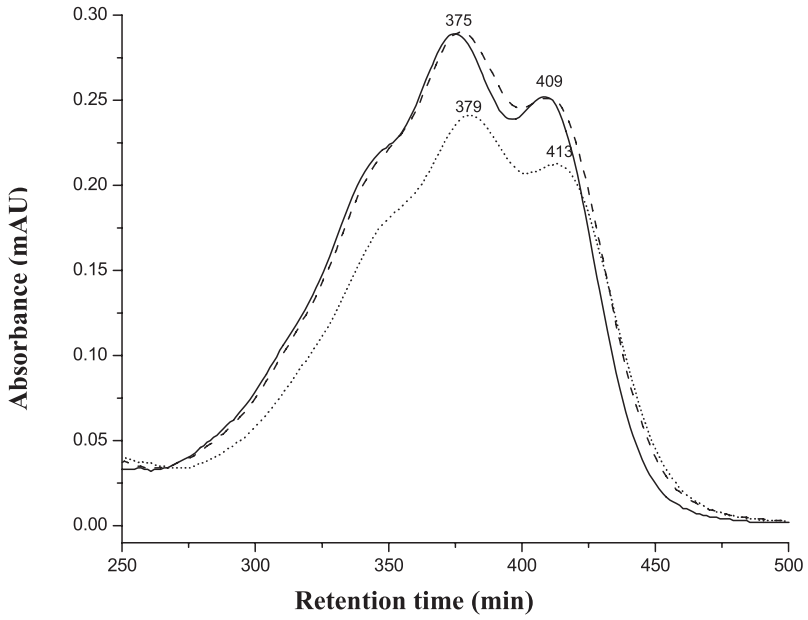


Fig. 5 The spectra of HPLC pigment pattern of KJ5 isolate at three different solvents; acetone (..); ethanol (--) and hexane (-) at 300-500 nm wavelengths.

isolate at three different solvents (Fig 5), and table 2 gives the absorption maximum of the pigments in various solvents.

Based on the HPLC analysis results that the maximum absorbance of *β*-carotene for this sample was in at a t_R 59.73 - 60.65 (Fig 4), the total *β*-carotene contents were calculated. The total *β*-carotene extracted from the *Erb. flavus* sample was 0,421 gr. The concentration of total *β*-carotene per gram of *Erb. flavus* can be calculated according to the following formula:

Yield (µg/mL) = 0.0108x + 12.677

(Limantara *et al*. 2013)

The concentration of total *β*-carotene per gram of wet weight of *Erb.flavus* is 30.01 g/g or 56.74 µg/ g of dry weight.

DISCUSSION

Erythrobacter flavus was proposed by Yoon *et al*., (2003). The characteristics of these bacteria are non-spore-forming rods, gram-staining reaction is negative, and motile by means of a single polar flagellum. Colonies are yellow, smooth, glistening, circular, convex with entire margins and 10-15 mm in diameter after 3 d cultivation at 30 °C on MA. Optimal temperature for growth is 30-37 °C. Growth occurs at 10 and 42 °C, but not at 4 °C or above 43 °C. Optimal pH for growth is 6.5-7.5 (Yoon *et al*. 2003). Most species of this genus contain bacteriochlorophyll α and carotenoids (Shiba and Simidu 1982; Yurkov *et al*. 1994).

The HPLC absorbant chromatogram is shown highlighting the separation of the pigment from *Erb.flavus*. UV-visible absorption spectra of carotenoid pigments are of immense importance, since they aid a great deal in determining the structure of carotenoids (Medicharla *et al*. 1991). From the HPLC profile and UV-visible absorption spectra, we can conclude that *Erb.flavus* produces a *β*-carotene type of carotenoid pigment that has many potential benefits.

From the data it was evident that *Erb.flavus* produced less than *β*-carotene as compared to other microorganisms e.g. *Dunaliella salina* (Prieto *et al*. 2011), fungi *Phycomyces blakesleeanus* (Murillo *et al*. 1978), and *Blakeslea trispora mutant* (*Mehta et al.* 1997). However, the *β*-carotene content reported in this study was higher than *Streptomyces* sp. which produced 4.88 µg per 100 gram (Baskar *et al*. 2010), *Erb.flavus* has been shown to have a potential strain for β-carotene production. The maximal *β*-carotene yield was 56.74 µg L^{-1} g of dry weight. These results suggest that strain KJ5 is worthy of further study for *β*-carotene industrialization.

Table 2. List of β-carotene spectral data from several data in the mobile phase and different solvents. where x is the broad absorption of the β-carotene, y is the concentration (µg mL^{-1})

References	Acetone	Ethanol	Hexane	Eluent
Britton *et al*. (1995)	-	425,450, 478	-	-
Jeffrey *et al*. (1997)	426,453,480 (III/II=21%)	427,449, 475	422,450,477 (III/II=36%)	425, 453, 476 (III/II=22%)
Hegazi *et al*. (1998)	-	-	425,449, 477	428,4 52, 476
Results	429,451,480 (III/II=20%)	429,454,479 (III/II=11%)	426,450,476 (III/II=33%)	427,449, 477 (III/II=13%)

Table 3 Broad absorption, yield and dry weight of β carotene

Broad absorption (x)	Y ield	Conc (µg/g dry weight)
1166. 17	12.64	56.74

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