

## Preliminary Investigation of the Carotenoid Composition of *Erythrobacter* sp. Strain KJ5 by High-Performance Liquid Chromatography and Mass Spectrometry

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**Separation and identification of carotenoids (Cars) from aerobic marine bacterium *Erythrobacter* sp. strain KJ5 are reported. The cells of *Erythrobacter* sp. were grown in a Shioi medium at 28.5° C for three days. Among the four solvents tested, the mixture of methanol and acetone (3:7, v/v) was determined as the optimum solvent for Car extraction from the cells by measuring its absorption spectrum. The Cars were separated via reversed-phase high-performance liquid chromatography using a C8 column and identified by a UV-Vis photodiode array detector and an electrospray ionization mass spectrometry. Bacteriochlorophyll *a* was not detected from the extracts of cells grown under both light and dark conditions. At least 16 peaks of Cars were separated, wherein eleven peaks showed the same absorption spectrum with  $\lambda_{\max}$  at 452-453 nm and at 478-480 nm. The other five peaks had an additional absorption peak at 340 nm, which belongs to *cis*-isomeric form. Two peaks of Cars were identified to be zeaxanthin and  $\beta$ -carotene.**

Key words:  $\beta$ -carotene, carotenoids, *Erythrobacter* sp., high-performance liquid chromatography, mass spectrometry, zeaxanthin

### INTRODUCTION

Carotenoids (Cars) are isoprenoid pigments in the form of yellow, orange, and red color found in both photosynthetic and non-photosynthetic organisms. Cars play a critical role in the photosynthetic process for higher plants, algae, and anoxygenic photosynthetic bacteria. Moreover, Cars act as photoprotector by quenching the chlorophyll triplet

and by scavenging singlet oxygen to protect cellular constituents i.e., DNA and membranes (Frank & Cogdell 1996). In addition, many evidences reveal the beneficial health effects of Cars as antiobesity (Miyashita 2009), antioxidant (Yeum et al. 2009), and anticancer (Kotake-Nara et al. 2001) agents.

Some Cars such as  $\beta$ -carotene,  $\alpha$ -carotene and  $\beta$ -cryptoxanthin serve as important sources of pro-vitamin A (Tang & Russell 2009). Moreover, in the food industry,

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Cars are mainly utilized as colorants; for example, annatto is responsible for the yellow to orange color of food commodities (Scotter 2009). Cars available in the market are generally produced by chemical synthesis. Nowadays, consumer demand drives the development of natural food colorants to replace the natural-identical colorings or synthetic colorants. Natural colorants are derived from natural sources like vegetable (e.g., carrot for  $\beta$ -carotene), fruit (e.g., tomato for lycopene), flower (e.g., stigma of *Crocus sativus* for saffron), fungus (e.g., *Blakeslea trispora* for  $\beta$ -carotene), and microalgae (e.g., *Dunaliella salina* for  $\beta$ -carotene) (Mortensen 2006). The other alternative source of natural Cars is bacteria due to the ability of bacteria to grow in a short time and controllable culture process. Since bacteria can be mass-produced, they are promising sources of Cars, thus avoiding large scale exploration of raw materials as well as chemically synthesized Cars (Sowmya & Sachindra 2015).

It is well stated that the majority of microbial species in nature, approximately more than 99% in some habitats, are uncultivated bacteria in the laboratory (Amann et al. 1995). In 1934, ZoBell and Feltham found yellow, orange, brown, and red bacteria colonies isolated from marine environments and at least 50% of marine bacteria have color. Many marine bacteria can synthesize a variety of Cars (Stafsnes et al. 2010). Recent study on a new Car from marine bacteria demonstrated that marine bacteria had potential for producing compounds with unique biological properties. Therefore, marine bacteria are promising potential sources of Cars (Shindo & Misawa 2014).

*Erythrobacter flavus*, a marine bacterium isolated from seawater of the east coast of Korea, had been proposed as a novel species by Yoon and co-authors (2003). Recently, Wusqy and co-workers (2014) isolated a yellow aerobic marine bacterium called strain KJ5 from hard-coral *Acropora nasuta* (Dana 1846) and reported that this bacterium had 96% similarity to *Erythrobacter flavus*. They also described  $\beta$ -carotene as a minor Car species, although there were several other dominant Cars present in *Erythrobacter* sp. strain KJ5. It was previously reported that *Erythrobacter* genus showed red, orange, or yellow colors and contained not only Cars, but also bacteriochlorophyll *a* (BChl *a*) (Shiba & Simidu 1982), although some species are lacking of BChl *a*.

The choice of extraction solvents depends on the biological material, sample treatment, and pigment composition (Schiedt & Liaaen-Jensen 1995). Water-miscible solvents are usually used for extraction of Cars, which have a broad polarity. Moreover, analysis of Cars (e.g., separation and identification) can be performed by reversed-phase high-performance liquid chromatography (RP-HPLC) equipped with diode array detector with

C-30 column (Rouseff et al. 1996). Cars have also been identified by liquid chromatography-mass spectrometry (LC-MS) using electrospray and atmospheric pressure chemical ionization with ionization methods as a powerful technique (van Breemen 1995; Rivera et al. 2014). Therefore, the objectives of this study are to determine the optimum solvent for Car extraction from the cells of *Erythrobacter* sp. strain KJ5 and to separate and identify their Cars by RP-HPLC combined with diode array detector and mass spectrometry (MS). The absence of BChl *a* in this bacterium is also described.

## MATERIALS AND METHODS

### Bacterium and cell culture

*Erythrobacter* sp. strain KJ5 isolated by Wusqy and colleagues (2014) was obtained from a culture collection of marine bacteria in Diponegoro University (Semarang, Indonesia). The cells were grown aerobically in an *Erythrobacter* medium reported by Shioi (1986) under illumination at light intensity of 21  $\mu\text{mole m}^{-2}\cdot\text{s}^{-1}$  to 26  $\mu\text{mole m}^{-2}\cdot\text{s}^{-1}$  (Apogee Instruments, Logan, US). The culture was carried out at 28.5° C for 72 h by shaking at 100 rpm (1 rpm = 1/60 Hz). The cells in the stationary phase were harvested by a centrifuge (Kubota, Tokyo, Japan) at 15 880 g and 4° C. The harvested cells were stored at -30° C until the next step.

### Carotenoid extraction

The cells of *Erythrobacter* sp. strain KJ5 (approximately 0.1 g wet weight) were disrupted by sonication for 10 min at 60% amplitude and 10-s on/30-s off pulse mode (Q500, QSonica, Newtown, CT. USA). Cars were then extracted by shaking for 5 min. Five different organic solvents that are commonly used for Car extractions – 100 % acetone, 100 % MeOH, 100 % hexane, mixtures of acetone and MeOH (7:3, v/v), and (3:7, v/v) – were tested to obtain maximum yield of the Cars in the presence of a small portion of  $\text{CaCO}_3$  and sodium ascorbate. The extracts were separated from the cell debris by centrifugation (Z 160 M, Hermle Labortechnik, Wehingen, Germany) at 8,000 g for 1 min and extraction was repeated five times. All extraction procedures were performed at low temperature (approx. 5° C) in a red light room. The crude Car extracts were combined and finally adjusted to 5 mL with the addition of the extraction solvent and then optimum Car extraction was determined by measuring its absorption spectrum (UV-1700 Spectrophotometer, Shimadzu, Kyoto, Japan). The Cars extracted with the optimum solvent was dried in a stream of  $\text{N}_2$  gas and stored at -30° C until used for HPLC analyses.

### Carotenoid saponification

Stepwise, 8.3 mL of methanolic solution of Car extracts, 1.7 mL of KOH (60%, w/v), and 10 mL of diethyl ether were added. The sample was gently bubbling with nitrogen and stirred for 60 min at room temperature under a dim light. The Car pigments were partitioned into diethyl ether by using NaCl saturated water. The diethyl ether phase was taken, dried under nitrogen, and kept at -30 °C for HPLC analysis.

### HPLC and LC-MS analyses

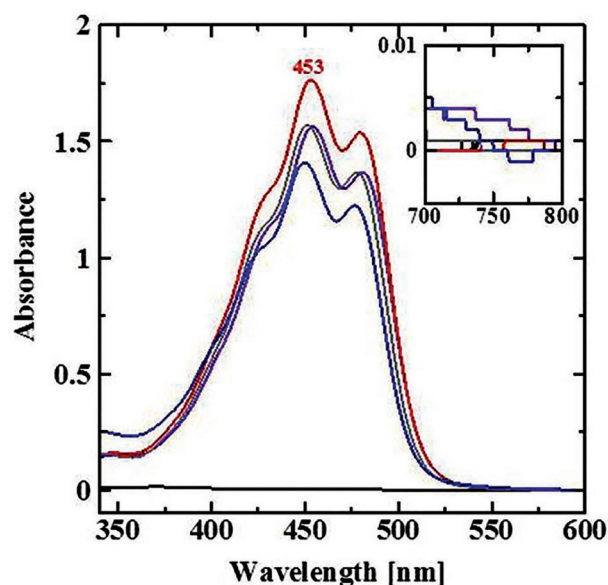
Separation, purification, and identification of Cars were performed by RP-HPLC with a UV-VIS photodiode array detector (Shimadzu, Kyoto, Japan) using the slightly modified method of Zapata and co-authors (2000). Mobile phase used for analysis comprised of eluent A – a mixture of methanol : acetonitrile : aqueous pyridine solution (0.25 M pyridine; 50:25:25, v/v/v) – and eluent B – methanol : acetonitrile : acetone (20:60:20, v/v/v) with a Symmetry C<sub>8</sub> column (150 x 4.6 ID mm, 3.5 μm particle size, 100 Å pore size) (Waters, Milford, USA). Pigments were separated at a flow rate of 1.0 mL/min at 30 °C with a programmed binary gradient elution system as follows: a linear gradient of 100% (A) to 60% (A) for 22 min, continued with a step to 5% (A) at 28 min, and finally isocratic elution of 5% (A) for 10 min. The elution was then returned to the initial conditions i.e., 100% (A) at 40 min. Identification of Cars was based on its spectroscopic and chromatographic properties – such as the position of absorption maxima ( $\lambda_{\text{max}}$ ), shape of absorption spectrum, and retention time – compared to the reference. Moreover, co-chromatography between crude Car extracts and Car standards, zeaxanthin, and  $\beta$ -carotene (NatChrom, Malang) was performed to evaluate the identity. Further, identification of Cars was carried out via electrospray ionization (ESI) triple quadrupole MS (LCMS-8030, Shimadzu, Kyoto, Japan) according to the modified method described by Brotosudarmo and colleagues (2017). Prior to LCMS analysis, each peak of Cars was isolated and purified by HPLC as described above.

LC-MS analysis was performed in a column of Cosmosil 2.5  $\mu$  NAP, 50 x 2.0 ID mm (Nacalai Tesque, Kyoto, Japan) with an isocratic elution of 0.1% formic acid in water (10%) and 0.1% formic acid in MeOH (90%) for 10 min at the flow rate of 0.3 mL min<sup>-1</sup>. The MS analysis was operated under the following conditions: (1) heat block temperature at 400° C; (2) desolvation line temperature at 250° C; (3) nebulizing gas (N<sub>2</sub>) flow at 3 L·min<sup>-1</sup>; (4) drying gas (N<sub>2</sub>) flow at 15 L·min<sup>-1</sup>; (5) interface (needle) voltage at 4.5 kV; (6) interface current at 0.1 μA; (7) mass range at 400–700 m/z; (8) ionization at positive and negative mode; and (9) collision energy at 10 V. To obtain precise data and information, the researchers analyzed the MS under both positive and negative ionization modes and

found that the positive mode was superior to the negative mode in terms of intensity of parent and fragment ions. Rivera and co-authors (2014) reviewed the analysis of carotenoids including  $\beta$ -carotene and zeaxanthin under the positive mode using ESI method.

## RESULTS AND DISCUSSION

It is known that most Cars are insoluble in water but soluble in organic solvents such as acetone, alcohol, tetrahydrofuran, ethyl ether, chloroform, and ethyl acetate and also easily soluble in petroleum ether, hexane, and toluene. On the other hand, xanthophylls dissolve better in methanol and ethanol (Rodriguez-Amaya 2001). However, the solubility of Cars generally depends on the presence of functional groups. To obtain optimum extraction and to examine solubility of Cars, cells were extracted with five different organic solvents with polar and non-polar property. Absorption spectra of the extracts with different organic solvents are shown in Fig. 1.



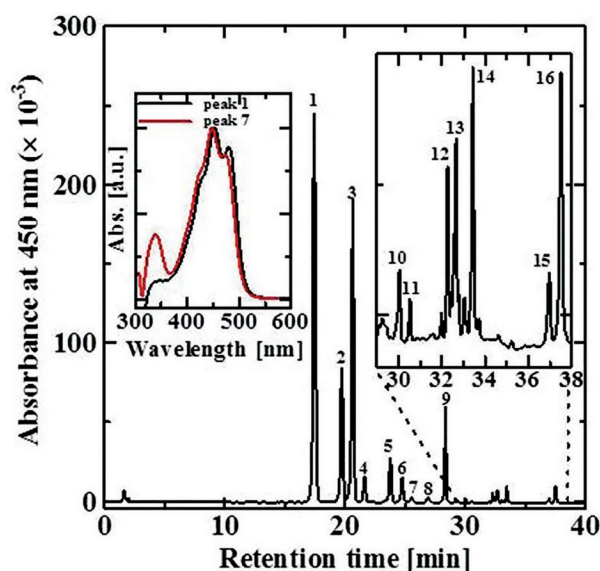
**Figure 1.** Absorption spectra of crude Car extracts from the cells of *Erythrobacter* sp. strain KJ5 extracted with 100 % hexane (black), 100 % acetone (violet), mixtures of MeOH and acetone (3:7, v/v) (red) and (7:3, v/v) (grey) and 100 % MeOH (blue). Inset: absorption spectra of crude Car extracts in the range of 700–800 nm.

Results showed that a mixture of MeOH and acetone (3:7, v/v) had higher intensity in absorption than any other solvents, indicating that this solvent is the most effective in Car extraction. Accordingly, hereafter this solvent was used for extraction throughout this study, unless otherwise noted. This solvent mixture is likely to use for the Car extraction from the cells like *Erythrobacter* sp. strain

KJ5, which comprises of mainly polar type of pigments. As shown in Fig. 1 (see black line), this is evident that hexane – a non-polar solvent – was unable to extract any pigments from the cells of this bacterium. The absorption spectrum of crude Car extracts from *Erythrobacter* sp. strain KJ5 showed a typical three-peaked shape of Car absorption, indicating vibrational fine structures. These  $\lambda_{\max}$  values were in accordance with the spectral property of zeaxanthin in acetone (–, 452, 479) (Britton 1995). This finding further suggests that the main Cars have a similar structure to zeaxanthin composed of nine conjugated double bonds at main skeleton and two cyclic end groups (Britton 1995).

Subsequently, it is reported that some *Erythrobacter* species only produce BChl *a* in the dark conditions (Shioi & Doi 1988). Therefore, the presence of BChl *a* in this bacterium was examined in the cells grown under dark in addition to light conditions. However, no absorption peak at around 770 nm (Limantara & Heriyanto 2011) was found in both extracts from cells grown under dark and light conditions (inset of Fig. 1). These results likely conclude that this bacterium does not produce BChl *a*.

The elution profile of crude Car extracts separated by RP-HPLC from the cells of *Erythrobacter* sp. strain KJ5 is shown in Fig. 2. In addition, the results of analysis by HPLC-diode array detector of each peak are summarized in Table 1. At least 16 peaks of Cars were well separated (Fig. 2), where 11 peaks had the same absorption spectrum



**Figure 2.** HPLC elution profile of crude Car extract from the cells of *Erythrobacter* sp. strain KJ5. Peak numbers in the elution profile correspond to those in Table 1. Insets: zoomed-in HPLC chromatogram of crude Car extract (right) and absorption spectra of peak 1 and peak 5 as representative of all *trans*-isomer and *cis*-isomer of Cars, respectively (left). The absorption spectra were normalized at  $\lambda_{\max}$ .

**Table 1.** Chromatographic and spectrophotometric properties of the Cars from *Erythrobacter* sp. strain KJ5 obtained by HPLC with a UV-Vis photodiode array detector.

| Peak No | $t_R$ [min] | $\lambda_{\max}$ [nm] | Car Identification        |
|---------|-------------|-----------------------|---------------------------|
| 1       | 17.4        | –, 452, 480           | esterified Car            |
| 2       | 19.8        | –, 453, 480           | esterified Car            |
| 3       | 20.8        | –, 452, 479           | Car                       |
| 4       | 21.7        | –, 452, 479           | Car                       |
| 5       | 23.9        | –, 452, 479           | Car                       |
| 6       | 24.9        | –, 452, 479           | Car                       |
| 7       | 25.6        | 340, –, 448, 471      | <i>cis</i> -isomer of Car |
| 8       | 27.0        | –, 452, 479           | Car                       |
| 9       | 28.5        | –, 453, 479           | Zeaxanthin*               |
| 10      | 30.1        | 340, –, 447, 471      | <i>cis</i> -isomer of Car |
| 11      | 30.5        | 340, –, 448, 472      | <i>cis</i> -isomer of Car |
| 12      | 32.2        | 340, –, 448, 472      | <i>cis</i> -isomer of Car |
| 13      | 32.6        | –, 452, 480           | Car                       |
| 14      | 33.4        | –, 452, 479           | Car                       |
| 15      | 36.7        | 340, –, 448, 472      | <i>cis</i> -isomer of Car |
| 16      | 37.2        | –, 453, 478           | $\beta$ -carotene*        |

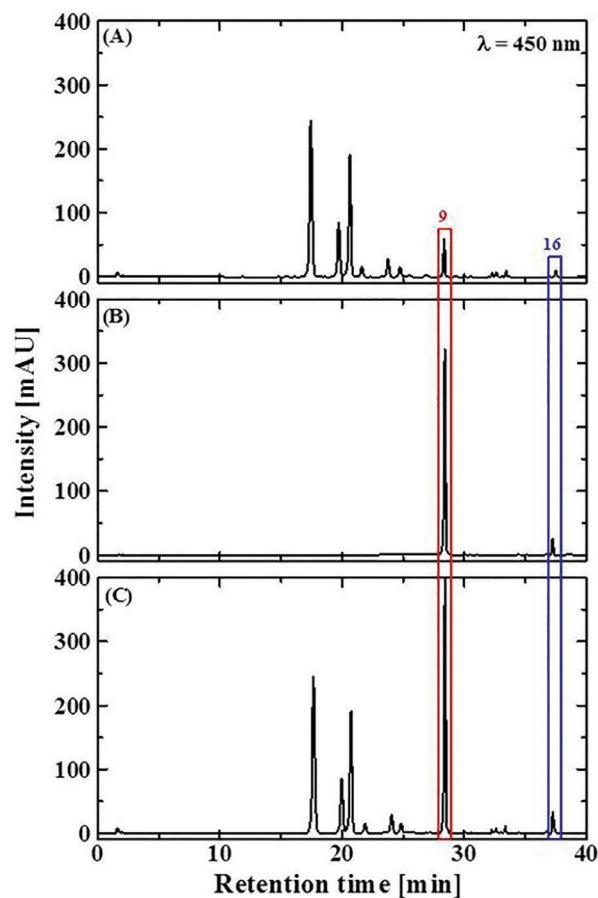
\*reference: Zapata and co-authors (2000)

with  $\lambda_{\max}$  at 452–453 nm and 478–480 nm, including three dominant Cars with retention times ( $t_R$ ) at 17.4 (peak 1), 19.8 min (peak 2), and 20.8 min (peak 3) (Fig. 2 and Table 1). The other five peaks had an additional absorption peak at 340 nm, which belongs to *cis*-isomeric form (Fiedor et al. 2016). In the spectrum of *cis*-isomers as shown in inset (left) of Fig. 2, a new peak appeared at around 340 nm, indicating significant changes in Car electronic structure due to the *cis* bend (Fiedor et al. 2016). Most Cars occur naturally as all *trans*-isomers. However, *trans* and *cis* conversion may take place during the extraction and isolation procedures (Schiedt & Liaaen-Jensen 1995). It is well known that *cis*-isomers are involved in photoprotection of reaction center, while all *trans*-isomers contribute in light harvesting (Koyama & Fujii 1999).

Maximum absorption of the pigments correlates to the numbers of conjugated double bond and also the type and number of additional modifications. Thus, Cars that have similar  $\lambda_{\max}$  have the same number of conjugated double bonds (Britton 1995). Moreover, another study showed that some Cars with different number of carbon atoms or different functional group do not have any effect on the absorption spectrum (Britton 1995). An additional conjugation in rings has less influence for maximum absorption (e.g., shift from 7 nm to 9 nm), whereas carbonyl functional groups increase  $\lambda_{\max}$  (Marasco 2008). As mentioned above, it is evident that eleven Cars have

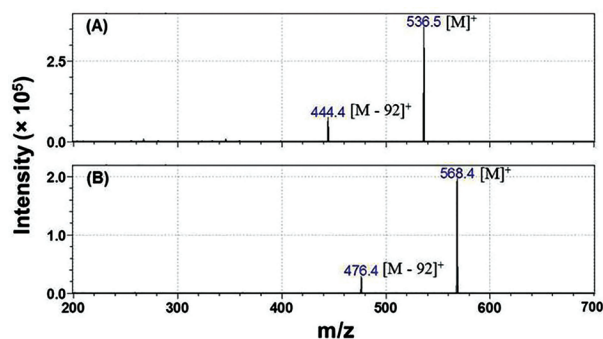
a similar core structure to zeaxanthin that comprises of nine conjugated double bonds at main skeleton and two cyclic end groups, but different  $t_R$  in their elution profiles by HPLC (Fig. 2). These facts further suggest that eleven Car molecules have different polarity and appear to have different numbers of functional group attached at the end group of Cars.

The results of co-chromatography using crude Car extracts and standard Cars, zeaxanthin, confirmed that peak 9 at 28.5 min correspond to zeaxanthin (Fig. 3). Similarly, peak 16 at 37.2 min was confirmed to be  $\beta$ -carotene by spiking of the peak with standard  $\beta$ -carotene. These Car identifications are supported from the chromatographic results reported by Zapata and co-workers (2000). Zeaxanthin and  $\beta$ -carotene have similar  $\lambda_{max}$ , but these Cars have different polarities as indicated by the different  $t_R$  values. Zeaxanthin has the same Car backbone as  $\beta$ -carotene, but with the addition of two hydroxyl groups at  $\beta$ -rings. The identification of these Cars was also



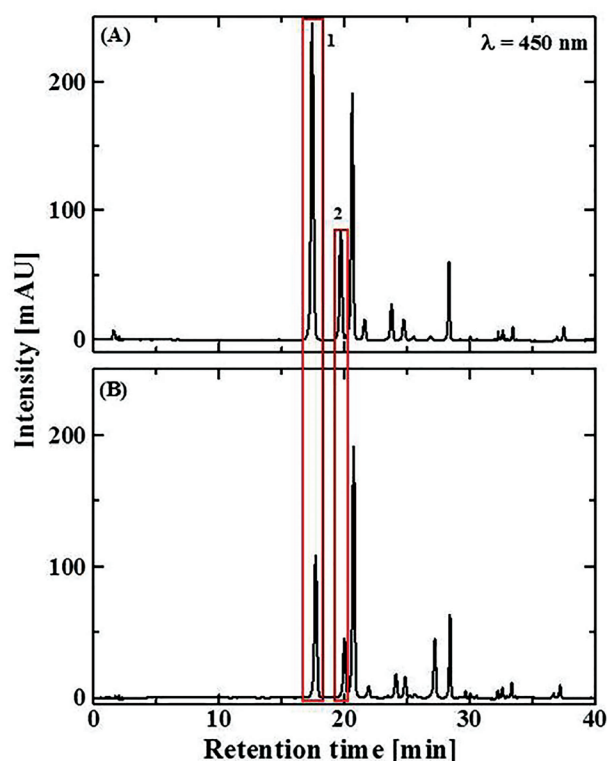
**Figure 3.** HPLC elution profiles of crude Car extract from the cells of *Erythrobacter* sp. strain KJ5 (A); a mixture of standards, zeaxanthin and  $\beta$ -carotene (B); Co-chromatography of crude Car extract with the standards, zeaxanthin and  $\beta$ -carotene (C). Peak 9 (red rectangle), zeaxanthin; and peak 16 (blue rectangle),  $\beta$ -carotene.

confirmed by the data from MS analysis. Mass spectrum of  $\beta$ -carotene showed  $[M]^+$  at  $m/z$  536.5 with fragment ion at  $m/z$  444.4  $[M - 92]^+$ , while the  $[M]^+$  and fragment ions of the zeaxanthin spectrum were at  $m/z$  568.4 and 476.4  $[M - 92]^+$ , respectively (Fig. 4). These fragment ions  $[M - 92]^+$  can be assigned to a loss of toluene ( $[M]^+ = 92.1$ ) from the parent ion as reported by Enzell and Back (1995). The mass spectra and fragment ions of zeaxanthin and  $\beta$ -carotene are in agreement with the results of Rivera and colleagues (2011) and Heriyanto and colleagues (2017).



**Figure 4.** MS spectra of  $\beta$ -carotene (A) and zeaxanthin (B) purified from the extracts of the cells of *Erythrobacter* sp. strain KJ5. Analysis was carried out positive mode under the conditions described in the text.

Three dominant Cars of *Erythrobacter* sp. strain KJ5 (peaks 1, 2 and 3) were eluted ahead of most of the elution peaks including that of zeaxanthin (Fig. 2). It indicates that these Cars have higher polarity than zeaxanthin, although their  $\lambda_{max}$  values were similar to zeaxanthin (Table 1). In the preliminary results of MS analysis, peak 1 and peak 2 showed higher  $m/z$  values and more signals due to fragment ions compared to those of peak 3. These features likely suggest that these peaks of Car are esterified form as reported by the results of saponification studies of crude Car extracts (Stafsnes et al. 2010; Zheng et al. 2013). Chromatographic results from saponified sample showed that these two peaks of Cars obviously diminished, while other peaks have a similar intensity as a non-esterified Car (Fig. 5). It is known that most marine bacteria produce  $C_{40}$ -type dicyclic Cars containing two  $\beta$ -end groups that are modified with keto and hydroxyl groups through xanthophyll biosynthetic pathways, with zeaxanthin as an intermediate (Gross 1991). Actually, astaxanthin, adonixanthin, erythroxanthin, and their derivatives including their esterified forms are ketolated and hydroxylated Cars from marine bacteria (Stafsnes et al. 2010; Misawa 2011; Zheng et al. 2013). Stafnsnes and colleagues (2010) identified nostoxanthin and the esterified nostoxanthin with sugar (diglucoside) as Car in *Erythrobacter* sp. (strain unknown). However, other *Erythrobacter* stains i.e., *Erythrobacter*



**Figure 5.** HPLC elution profiles of crude Car extract from the cells of *Erythrobacter* sp. strain KJ5 before (A) and after (B) saponification treatment. Note that after saponification (B), peaks 1 and 2 in red rectangles were obviously diminished.

sp. NAP1, *E. longus* DSM 6997, *E. litoralis* T4T, *Erythrobacter* sp. JL475, produce erythroanthin sulfate, bacteriorubixanthin, zeaxanthin, and  $\beta$ -carotene (Zheng et al. 2013). Therefore, Car identification in *Erythrobacter* sp. strain KJ5 needs further studies that must be carried out with sophisticated methods and instruments.

## CONCLUSIONS

Yellow aerobic marine bacterium *Erythrobacter* sp. strain KJ5 did not contain BChl *a*, but it had at least 16 Car species with a similar absorption property of zeaxanthin and its isomers. A mixture of MeOH and acetone (3:7, v/v) was the most effective for Car extraction. Most of the Cars of this bacterium have a similar core chemical structure. In this study, two peaks of Cars were identified as zeaxanthin and  $\beta$ -carotene by HPLC and ESI-MS.

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