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CAROTENOID PRODUCTION FROM SPONGE-ASSOCIATED BACTERIA ISOLATED IN THE GULF OF THAILAND

Patcharee Thawornwiriyanun[1], Chutiwan Dechasakulwatana[2],
Leena Suntornsuk[3], and Worapot Suntornsuk[1]*

[1]*Department of Microbiology, Faculty of Science, King Monkut's University of Technology Thonburi, Bangkok 10140, Thailand.*
[2]*Institute of Marine Science, Burapha University, Bangsaen, Chonburi 20131, Thailand.*
[3]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand.*

ABSTRACT

This research was aimed at identifying a sponge-associated bacterium that produces carotenoids and studying optimum conditions for carotenoid production. Bacterial identification was carried out by the determination of their shape, size, colony's color, motility, growth on semisolid medium, Gram staining, and a rapid biochemical test kit, API 20 NE. The results showed that one of the sponge-associated bacteria producing carotenoids was identified as *Methylobacterium mesophilicum* MAKB08-4. It produced astaxanthin as a main pigment of carotenoids analyzed by HPLC. It had an optimum astaxanthin production condition at a pH of 7, a temperature of 25°C, and an agitation rate of 100 rpm with 1% (w/v) glucose as a carbon source and the absence of NaCl addition in the Modified Zobell broth. Under the optimal condition, it had a good growth of 0.6 g/l and produced astaxanthin at 0.67 mg/l. The strain would be a carotenoid source, especially for astaxanthin applied for the food, cosmetic and pharmaceutical industries.

Keywords: Sponge-associated bacteria, carotenoid, production, *Methylobacterium mesophilicum.*

INTRODUCTION

Carotenoids are important orange-yellow pigments popularly used as additives in food, feed, cosmetic and pharmaceutical products. They can also serve as antioxidants preventing free radical formations. They are commonly found in natural products, such as vegetables and fruits. Moreover, carotenoids, such as β-carotene, zeaxanthin, canthaxanthin, rhodoxanthin, and astaxanthin, can be produced by molds such as *Blakeslea trispora*; yeast, i.e., *Rhodotorula* sp. and *Phaffia rhodozyma*; algae, for example, *Dunaliella* sp. and *Haematococcus pluvialis*; and bacteria such as *Pseudomonas rodos, Flavobacterium* sp. and *Micrococcus* sp. (Goodwin, 1980; Margalith, 1992; Vandamme, 1994). Recently,

*Corresponding author. E-mail address: worapot.sun@kmutt.ac.th

colorful sponge-associated bacteria have been isolated from the Gulf of Thailand. They have a distinctive color range of orange to yellow. However, they have never been investigated for carotenoid production.

The objectives of this research were to identify a sponge-associated bacterium producing carotenoids and to determine optimum conditions for carotenoid production by the bacterium.

MATERIALS AND METHODS

Materials

The Institute of Marine Science, Burapha University, Chonburi, Thailand kindly provided seawater. It was filtered through a Whatman No.4 filter paper and kept at 4°C before it was used. Standard pigments used, β-carotene and astaxanthin, were purchased from Sigma- Aldrich, (St. Louis, MO, U.S.A.).

Microorganisms and cultivation medium

Twenty four sponge-associated bacteria were kindly provided by the Institute of Marine Science, Burapha University. Each bacterium was kept in a modified Zobell medium slant containing 1 g of yeast extract, 1 g of proteose peptone (BBL), 0.5 g of phytone peptone (BBL), 0.2 g of sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$), 0.05 g of sodium sulfite (Na_2SO_3), and 0.2 g of ferric citrate ($Fe(C_6H_5O_7)$) in 1 liter of seawater and distilled water (90:10 v/v) at 4°C before use.

Bacterial morphology study and identification

Bacterial cells were observed by Gram's staining under light microscopy. Their color, growth, and mobility on modified Zobell medium were also monitored. A bacterial strain expressing the highest carotenoid production was further identified by an API test kit (API 20 NE, BioMerioux, France). The results were analyzed by using Apiweb program.

Carotenoid production

Selected bacterial strains were cultivated in flasks containing 100 ml of the modified Zobell medium. The cultivation was carried out at 20°C and 100 rpm for 4 days. Bacterial growth and carotenoid production were determined.

Optimal conditions of carotenoid production

The best carotenoid-producing strain was cultured in flasks containing 100 ml of the modified Zobell medium for 4 days. Cultivation conditions studied were pH (6, 7, and 8), agitation rate (100, 150, and 200 rpm), incubating temperature (25, 30, and 35°C), glucose concentration (0, 1, and 2 %w/v), and NaCl supplement (0, 2 and 4%w/v). Bacterial growth and carotenoid production were determined after 4 days of cultivation.

Carotenoid extraction and preparation

Bacterial cells were harvested by centrifugation at 6,000 x g, 4°C for 15 min and washed twice with distilled water. The cell pellet was suspended in 95% (v/v) ethanol and the cell suspension was shaken at 200 rev/min at 30°C for 24 h. Cell debris was removed by centrifugation at 6,000 x g, 4°C for 15 min and ethanol extract was then collected. The extract was evaporated to dryness by nitrogen gas and re-dissolved in acetone. The extract was filtered through a 0.45 mm membrane before HPLC analysis.

Analyses

Bacterial growth was determined by an optical density measurement at 600 nm. The growth was also determined by cell dry weight measurement. After bacterial cultivation, cells were collected by centrifugation at 6,000 x g, 4°C for 15 min and washed twice with distilled water. The cell pellet was then dried at 60°C until constant weight.

Carotenoids in the extract were measured by a spectrophotometric method at 480 nm (Goodwin, 1976). Carotenoids were also analyzed by reverse phase HPLC using a Polaris C18-A column (150 x 4.6 mm) connected with a guard column (20 x 4.6 mm). Acetone:hexane (10:90, v/v) was used as an elution system at the flow rate of 0.5 ml/min. Carotenoids in the samples were detected by a

photodiode array detector at 450, 462, and 471 nm at a room temperature. β-carotene and astaxanthin were used as external standards.

RESULTS AND DISCUSSION

Bacterial morphology

Isolated sponge-associated bacteria had various characteristics as shown in Table 1. All strains were Gram negative except for MAKB08-3 and MAKB19-3. Similarly, most bacteria associated with *Petrosia ficiformis* and *Suberites domuncula* sponges found were Gram negative (Chelossi et al., 2004; Osinga et al., 2001). Most isolated bacteria were non-motile and rod shapes with differences in size (0.6-6.0 mm). They had especially a wide color range of yellow to orange. Therefore, they were expected to produce carotenoids.

Table 1. Morphological characteristics of isolated sponge-associated bacteria.

Strain	Color	Mobility	Gram stain	Growth*	Shape	Size (mm)	
						Length	Width
MAKB 05-2	Orange-pink	+	Negative	++	Rod	3.2-4.3	0.91-0.95
MAKB 07-1	Brown	-	Negative	+++	Rod	5.2-6.2	1.12-1.15
MAKB 08-3	White	+	Positive	++	Cocci	0.9-1.4	
MAKB 08-4	Yellow	+	Negative	+++	Short rod	2.1-2.4	1.0-1.2
MAKB 13-3	Pale orange	+	Negative	+++	Rod	3.4-3.6	1.1-1.9
MAKB 19-3	Yellow	-	Positive	++	Cocci	1.3-1.9	
MAKB 19-4	Red	-	Negative	++	Short rod	1.1-1.2	0.5-0.56
KODA 11-3	Yellow	-	Negative	+++	Short rod	2.3-2.6	0.73-0.79
KODA 16-3	White	-	Negative	++	Rod	3.8-4.1	1.65-1.9
KODA 19-6	Yellow	+	Negative	++	Cocci	1.3-1.7	
CHOB 04-1	Pale orange	+	Negative	++	Rod	3.7-4.2	1.28
CHOB 04-4	Yellow	-	Negative	++	Short rod	1.9-1.95	0.91
CHOB 05-4	Yellow	-	Negative	+	Cocci	0.6-0.7	
CHOB 06-6	Yellow	-	Negative	++	Short rod	2.2-2.3	0.9-1.1
CHOB 09-1	Brown	-	Negative	++	Rod	4.2-4.6	1.39
CHOB 10-1	Red	-	Negative	++	Short rod	0.96-1.25	0.5-0.7
CHOB 14-2	Brown	-	Negative	++	Cocci	1.1-1.5	
CHOB 15-2	Pale yellow	-	Negative	++	Short rod	1.1-1.5	0.4-0.43
SAMD 03-1	White	-	Negative	+	Rod	4.0-4.6	0.9-1.1
SAMD 03-4	Orange	+	Negative	++	Cocci	1.1-1.4	
SAMD 05-4	Orange Yellow	-	Negative	+	Rod	2.8-3.5	0.9-1.1
SAMF 04-2	Pale yellow	-	Negative	+	Short rod	1.7-1.8	0.4-0.5
SAMF 04-7	White	-	Negative	+	Short rod	1.1-1.2	0.6-0.7
SAMO 04-1	Pale orange	+	Negative	++	Rod	2.8-2.9	0.9-1.0

*on Modified Zobell semi solid medium
* - non-motile, + motile
** - Gram negative, + Gram positive
*** on Modified Zobell semi solid medium: + moderate, ++ good, +++ very good

Carotenoid production

Colorful isolated strains were selected for carotenoid production by flask cultivation. Their carotenoid production and growth are shown in Table 2. The bacteria grew well in the modified Zobell medium. The strain SAMD 03-4 gave the highest growth. It, however, produced only a small amount of carotenoids. These bacteria produced carotenoids in a range of 0.14-0.51 mg/l. They produced much lower carotenoids than *Rhodobacter sphaeroides* previously reported by Chen et al. (2006) due to not only dissimilar bacterial strains but also different medium compositions and cultivation conditions employed. However, the strain MAKB 08-4 was found to be the highest carotenoid producer. It was, therefore, selected for further studies of its bacterial identification and optimal condition for carotenoid production.

Table 2. Growth and carotenoid production of selected sponge-associated bacteria.

Strain	Growth (OD 600 nm)	Carotenoid concentration (mg/l)
MAKB 19-3	0.672	0.32 ± 0.06
CHOB 06-6	0.224	0.43 ± 0.03
SAMD 03-4	1.535	0.21 ± 0.04
MAKB 08-4	0.214	0.51 ± 0.07
MAKB 05-2	0.934	0.17 ± 0.02
KODA 11-3	0.372	0.14 ± 0.04
KODA 19-6	0.563	0.42 ± 0.05

Bacterial identification

The strain MAKB08-4 was identified as *Methylobacterium mesophilicum* by the API 20 NE test kit with 81% identity. *Methylobacterium mesophilicum* or *Pseudomonas mesophilica* was firstly isolated from the surface of green leaves of *Lolium perenne* or perennial rye grass (Austin and Goodfellow, 1979). It was also found as an endophyte from branches of sweet orange (*Citrus sinensis* Osbeck cv. Natal) (Araújo et al., 2002). According to Lidstrom and Chistoserdova (2002), Methylobacteria were classified as α-Proteobacteria and had twenty three species found. They are Gram-negative, rod shaped and obligately aerobic. These bacteria, generally, show pink-pigmented colonies which is a primary diagnostic characteristic for initial isolation of Methylobacteria. They can use methanol, methylamine and C_2, C_3 and C_4 compounds as carbon sources. They are commonly found in soil and on surfaces of leaves and other plant parts. It is interesting that our strain MAKB08-4 showed some different characteristics from Methylobacteria in the literature possibly because of different cultivation medium and conditions and its habitat. To our knowledge, it is the first report that *Methylobacterium mesophilicum* was found associated with a sponge.

Carotenoid analysis

HPLC chromatogram of the extract from the strain MAKB08-4 is shown in Figure 1. It was found that carotenoids mostly produced by the strain MAKB08-4 were astaxanthin. The strain may also produce astaxanthin derivatives or other forms of carotenoids. This is the first work to show a sponge-associated bacterium produced astaxanthin, especially *Methylobacterium mesophilicum*. The bacterial cells would be an alternative natural source of astaxanthin as found in cells of yeast *Phaffia rhodozyma* or microalgae *Haematococcus pluvialis*. This pigment is a high value chemical and generally used as a

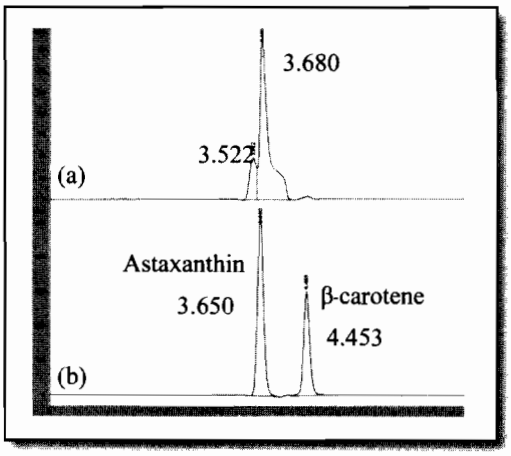

Figure 1. HPLC Chromatograms: (a) the extract from MAKB08-4 (b) astaxanthin and β-carotene standards.

supplement in animal feeds to improve red color in animal tissues and to increase reproduction rates and immunity in animals (Krinski, 1991).

Optimal conditions of astaxanthin production

The results of pH of medium affecting astaxanthin production by the strain MAKB08-4 are shown in Table 3. The pH of medium slightly affected its growth and astaxanthin production. However, the highest astaxanthin yield (0.53 mg/g dry cell weight) was accomplished when the strain was cultivated in the medium pH of 7.0. It is possible that this pH is optimal for the enzymatic reactions of its carotenoid production. The result was similar to astaxanthin production from the microalga *Haematococcus pluvialis* (Sarada et al., 2002). The report showed that both growth and astaxanthin contents of *Haematococcus pluvialis* were maximal at pH 7.0. However, the optimal pH for astaxanthin production from the yeast *Phaffia rhodozyma* was 6.0 (Ramírez et al., 2001).

Table 3. Effect of pH on astaxanthin production by the strain MAKB08-4. Cultivation condition: agitation rate at 150 rpm and temperature at 30°C.

pH	Growth (g/l)	Astaxanthin (mg/l)	Astaxanthin yield (mg/g)
6	0.4	0.20 ± 0.02	0.50
7	0.4	0.21 ± 0.01	0.53
8	0.5	0.21 ± 0.02	0.42

Agitation rate highly affected astaxanthin production from the bacterium as shown in Table 4. The strain MAKB08-4 produced the highest astaxanthin (0.30 mg/l) when it was shaken at 100 rpm in the medium (pH 7.0) at 30°C. Agitation generally influences bacterial product formation since it provides homogenous nutrient and oxygen distribution in a container. A higher agitation rate negatively affected astaxanthin production by the bacterium as it might cause a high shear rate unsuitable astaxanthin production. It is interesting that *Agrobacterium aurantiacum*, an astaxanthin-producing bacterium, was also able to produce a good yield of astaxanthin at 100 rpm shaking speed (Yokoyama et al., 1994). However, during cultivation of *Haematococcus pluvialis*, astaxanthin accumulation in its cells with no aeration was higher than their pigment accumulation with aeration (Domínguez-Bocanegra et al., 2007).

Table 4. Effect of agitation rate on astaxanthin production by the strain MAKB08-4. Cultivation condition: pH of 7 and temperature at 30°C

Agitation rate (rpm)	Growth (g/l)	Astaxanthin (mg/l)	Astaxanthin yield (mg/g)
100	0.4	0.30 ± 0.02	0.75
150	0.5	0.23 ± 0.01	0.46
200	0.5	0.23 ± 0.01	0.46

Temperature highly affected the growth and astaxanthin production by the strain MAKB08-4 (Table 5). The bacterium produced high astaxanthin (0.35 mg/l) when it was incubated at 25°C. Temperature would control the enzymatic reactions in bacterial carotenogenesis pathway (Bhosale, 2004). Moreover, this temperature might be the same or nearest temperature of the sponge which was the host of the bacterium. Ramírez et al. (2001) found that *Phaffia rhodozyma* produced the highest astaxanthin at 19.7°C. However, the green alga, *Chlorococcum* sp., was found to produce astaxanthin at the optimal temperature at 35°C (Liu and Lee, 2000).

Table 5. Effect of temperature on astaxanthin production by the strain MAKB08-4. Cultivation condition: pH of 7 and agitation rate at 100 rpm

Temperature (°C)	Growth (g/l)	Astaxanthin (mg/l)	Astaxanthin yield (mg/g)
25	0.65	0.35 ± 0.01	0.54
30	0.5	0.24 ± 0.00	0.48
35	0.4	0.22 ± 0.00	0.55

Glucose was added to be a carbon and energy source in the modified Zobell medium. It considerably affected astaxanthin production, as shown in Table 6. Addition of 1% glucose gave good growth and the highest astaxanthin production at 0.6 g/l and 0.67 mg/l, respectively. Glucose would play the important role in astaxanthin production since it is a precursor for glycolysis pathway which leads to formation of acetyl CoA, the starting substance for isoprenoid synthesis. Excess glucose concentration would lead to the feedback control of the metabolic pathway and reduce astaxanthin productivity. Ip et al. (2003) reported that the green microalga *Chlorella zofingiensis* produced the maximal astaxanthin in the medium containing 30 g/l glucose. *Phaffia rhodozyma* mutant HT-5FO1C was reported to produce astaxanthin in the optimal medium containing 25 g/l glucose (An et al., 1996).

Table 6. Effect of glucose concentration on astaxanthin production by the strain MAKB08-4. Cultivation condition: pH of 7, agitation rate at 100 rpm and temperature at 25°C

Glucose (% w/v)	Growth (g/l)	Astaxanthin (mg/l)	Astaxanthin yield (mg/g)
0	0.5	0.27 ± 0.18	0.54
1	0.6	0.67 ± 0.15	1.12
2	0.7	0.55 ± 0.02	0.79

NaCl supplement in the medium strongly affected the astaxanthin production as shown in Table 7. The medium without NaCl addition which contained 3.2% salinity was suitable for astaxanthin production. High salinity would be the stress for bacterial cells by lowering water activity (a_w) in the medium. Microalgae *Haematococcus pluvialis* was reported to produce high astaxanthin when it was cultivated in the medium containing 0.25 - 0.5% NaCl (Sarada et al., 2002). Astaxanthin production declined when 1% NaCl was added into the medium and microalgal cells were bleached after 2% NaCl was added (Sarada et al., 2002).

In addition, *Agrobacterium aurantiacum*, a marine bacterium, isolated from the surface of the sea in the Okinawa area of Japan was found to produce astaxanthin of 0.11 mg/l (Yokoyama and Miki, 1995). It produced much fewer astaxanthin than the strain MAKB08-4 at the optimal condition (Table 7). However, compared to the bacterial strains, an astaxanthin-producing yeast, *Phaffia rhodozyma (Xanthophyllomonas dendrorhous)*, produced a significantly higher level of astaxanthin at 8.1 mg/l (Ramírez et al., 2001).

Table 7. Effect of NaCl supplementation on astaxanthin production by the strain MAKB08-4. Cultivation condition: pH of 7, agitation rate at 100 rpm, temperature at 25°C and 1% glucose addition

NaCl (% w/v)	Growth (g/l)	Astaxanthin (mg/l)	Astaxanthin yield (mg/g)
0	0.6	0.67 ± 0.15	1.12
2	0.5	0.20 ± 0.01	0.40
4	0.5	0.16 ± 0.01	0.32

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