Polish Journal of Microbiology
2014, Vol. 63, No 1, 115–119

SHORT COMMUNICATION

Zeaxanthin Biosynthesis by Members of the Genus *Muricauda*

SUDHARSHAN PRABHU, P.D. REKHA and A.B. ARUN*

Yenepoya Research Centre, Yenepoya University, Deralakatte, Mangalore, Karnataka State, India

Submitted 3 March 2013, revised 3 May 2013, accepted 16 November 2013

Abstract

Zeaxanthin, a C_{40} xanthophyll carotenoid, has potential biological applications in nutrition and human health. In this study we characterized carotenoid composition in 5 taxonomically related marine bacterial isolates from the genus *Muricauda.* The pigment was characterized using high performance liquid chromatography (HPLC) and mass spectrometry, which confirmed the presence of all-trans-zeaxanthin. *Muricauda* strains produced zeaxanthin as a predominant carotenoid. *M. flavescens* JCM 11812^T produced highest yield (4.4 ± 0.2 mg L^{-1}) when cultured on marine broth at 32°C for 72 h. This is the first report on the presence of zeaxanthin among the majority of species from the genus *Muricauda*.

Key words: *Flavobacteriaceae*, *Muricauda*, marine bacteria, zeaxanthin

Zeaxanthin is a potential biomolecule having antioxidant, anticancer properties and is known to prevent age related macular degeneration (Krinsky *et al*., 2003). Apart from plant sources, microbes have been found as an important source of carotenoids particularly zeaxanthin (Hameed *et al*., 2011). Though many bacteria are known to produce zeaxanthin, the major limitation for their use in large scale commercial exploitation is that they often produce mixed carotenoids similar to plant source. Separation of zeaxanthin from total carotenoids involves multiple purification steps and turns out to be expensive. Selection of strains that grow faster and accumulate high amount of zeaxanthin can facilitate efficient extraction and purification processes. Isolation of bacteria producing biocompatible natural zeaxanthin as the major carotenoid in higher concentration will be vital for meeting large scale demand. Most important members that were reported earlier for the production of zeaxanthin are *Flavobactrium multivorum, Mesofalvibacter zeaxanthinfaciens, Zeaxanthinibacter enoshimensis, Muricauda lutaonensis* and recently described *Siansivirga zeaxanthinfaciens (*Asker *et al*., 2007a; b; Hameed *et al*., 2011; 2012). However, zeaxanthin production dynamics in closely related strains or species have not been studied.

Muricauda is a genus under the family *Flavobacteriaceae* (Bruns *et al*., 2001) of marine origin. It currently encompasses seven type strains known to produce characteristic orange-yellow pigmented colonies (Lee *et al*., 2012; Arun *et al*., 2009; Hwang *et al*., 2009; Lee *et al*., 2012). *M. lutaonensis* CC-HSB-11^T isolated from a coastal hot-spring was first reported to produce high amounts of zeaxanthin (Hameed *et al*., 2011). However, no other details are available for zeaxanthin production pertaining to other species in this genus. Hence, this study was undertaken to investigate the production of zeaxanthin by other members of the genus *Muricauda*.

Four type strains of the genus *Muricauda*, namely, *M. aquimarina* JCM 11811^T, *M. flavescens* JCM 11812^T, *M. lutimaris* KCTC 22173^T and *M. lutaonensis* KCTC 22339^T and two environmental isolates, YUAB-SO-11 and YUAB-SO-45 isolated from Ullal and Cochin beaches, South-West coast of India respectively were used. All strains otherwise indicated were sub-cultured on marine agar 2216 (Difco) at 32°C, whereas, *M. lutaonensis* at 42°C and stored in 30% glycerol at –80°C. The two environmental isolates YUAB-SO-11 and YUAB-SO-45 were identified using 16S rRNA gene sequencing according to the method described earlier (Kämpfer *et al*., 2003). Sequence data was analyzed after multiple sequence alignment. Sequences were submitted to GenBank (GenBank Accession Number: YUAB-SO-11, JQ257008 and YUAB-SO-45, JQ346699). Optimum growth and carotenoid production of the strains was determined. Approximately 10^8 CFU mL^{-1}

* Corresponding author: A.B. Arun, Deputy Director, Yenepoya Research Center Yenepoya University, Deralakatte, Mangalore- 575018, Karnataka State, India; phone: +91 824 2204668/969/70 ext. 302; mobile: +91 9980856605; fax: +91 824 2204673, **e-mail: bhagwatharun@hotmail.com; dydirectoryrc@yenepoya.edu.in**

were inoculated into 100 mL flask containing 20 mL marine broth 2216 (MB) and incubated at 32°C, in the case of *M. lutaonensis* at 40°C, under shaking at 150 rpm for 120 h. Cell density was determined at 24 h intervals by reading OD_{600} using an UV-Visible spectrophotometer (Shimadzu 1800). Cell dry weight (CDW) was determined by centrifuging 1 mL of the broth culture at 7500 rpm in a pre-weighed vial and the pellet was washed twice in sterile water and dried at 80°C to constant weight. Characterization and quantification of the carotenoid was carried out as outlined by (Asker *et al*., 2007c; Hameed *et al*., 2011; Sanusi and Adebiyi, 2009). 10 mg of the lyophilized biomass was suspended in 10 mL ethanol and incubated overnight at 50°C in dark with agitation for carotenoid extraction. The carotenoid in solution was separated from the biomass by centrifugation (12000 rpm for 10 minutes at 4°C). This step was repeated until complete extraction. The solvent was evaporated under nitrogen gas in the dark and re-dissolved in 1 mL of ethanol and subjected to a full-wavelength scan (250–700 nm) using a UV-Visible spectrophotometer (UV-Vis 1800, Shimadzu, Japan). The peak obtained was plotted against calibration curve constructed using standard zeaxanthin.

Purification and identification of the carotenoid was carried out according to the methods outlined earlier using HPLC (Asker *et al*., 2007). The HPLC system with a diode array detector (L-2455, Hitachi) attached to RP column (CAPCELL PAK C18 MG S-5, 35 × 4.6 mm, 5 µm particle size) maintained at 35°C, coupled to a HPLC pump (L-2130, Hitachi) was used. Identification of the HPLC purified carotenoid was carried out using mass spectrometry consisting of a linear ion trap mass spectrometer (Thermo LTQ XL, USA) attached to a LC plus system (Thermo Scientific). Conditions (ion source, atmospheric pressure chemical ionization (APcI) source, operated in the positive ion mode; sheath gas flow (N_2), 50 arbitrary units; auxiliary gas flow (N_2), 10 arbitrary units; source voltage, 6 kV; capillary temperature, 300°C) were maintained. Full mass scan was selected with a separation width of 2 *m*/*z* unit for collision-induced dissociation with the collision energy of 25 eV for detection. The carotenoids were identified and confirmed using standard zeaxanthin. All the strains used in this study showed the presence of zeaxanthin exclusively.

The phylogenetic analysis of the *Muricauda* type strains and two environmental isolates along with the type strains of the genus *Muricauda* is given in Figure 1. The 16S rRNA gene sequence similarity in the genus *Muricauda* ranged between 95.4–99.2%. Identification of the two isolates based on 16S rRNA gene sequences revealed that both the isolates YUAB-SO-11 and YUAB-SO-45 (Fig. 1) belonged to the genus *Muricauda*. Strain *Muricauda* sp. YUAB-SO-11 was closely

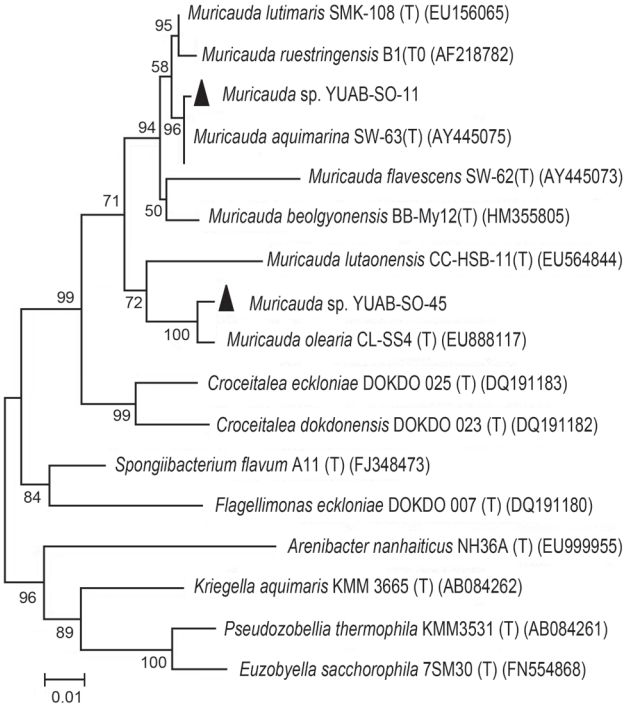

Fig. 1. Phylogenetic analyses based on 16S rRNA gene sequences showing the relationship between two recently isolated environmental strains, *Muricauda* sp. YUAB-SO-11 (Accession Number: JQ257008) and *Muricauda olearia* YUAB-SO-45 (Accession Number: JQ346699) to other members of genus *Muricauda.* Distances and clustering with the neighbor joining method were determined by using the software package MEGA version 5. Bar, 0.01 substitutions per nucleotide.

related to *M. aquimarina* JCM 11811^T (98.9% similarity) but could be distinguished clearly from the type strain *M. aquimarina* JCM 11911^T in the phylogenetic analysis indicating that strain YUAB-SO-11 might represent a novel species in the genus *Muricauda*. The strain *Muricauda* sp. YUAB-SO-45 was closely related to *M. olearia* (JCM 15563^T) (99.2% similarity). Physiological and biochemical characterization of the *Muricauda* strains is provided in the Table I. Strain *Muricauda* sp. YUAB-SO-11 utilized only glucose and could not utilize other carbon sources. The maximum biomass produced by different strains is listed in Table II. Among the strains, *M. flavescens* JCM 11812^T showed the highest cell biomass yield (2.9 ± 0.1 g L^{-1}) in marine broth, whereas, strain *M. lutaonensis* KCTC 22339^T showed comparatively lower biomass (1.9 ± 0.1 g L^{-1}) at 72 h of incubation in marine broth. Optimum growth and carotenoid production for all the strains was at pH 7–8 and 2–3% salinity (NaCl) and temperature 30–35°C except for the strain *M. lutaonensis* KCTC 22339^T, which was 40°C. Preliminary spectrophotometric analysis of the ethanol-diluted crude carotenoid from all the *Muricauda* strains showed typical absorption spectra with a λ_{max} at 448 nm with shoulder peaks identical to those of the standard all-*trans*-zeaxanthin.

Table I
Differential physiological and biochemical characterstics of the genus *Muricauda*

Characteristics		1	2	3	4	1	1
Growth temperature (°C)	Range	30–38	25–38	10–44	25–35	30–55	30–39
	Optimum	32	32	30–37	30–32	37–45	30–35
Growth pH	Range	5–9	5–9	5–9	5.2–9.4	6–9	5–9
	Optimum	7–8	7–8	7.6	6.8–7.7	7.8	7–8
Growth with NaCl (%)	Range	2–10	2–10	2–9	2–6	2–6	1–10
	Optimum	2	2	2	2–3	3–5	2–3
Gliding motility		–	–	–	–	–	–
Spore formation		–	–	–	–	–	–
Enzyme activity	Oxidase	+	+	+	+	+	+
	Catalase	+	+	+	+	+	+
Nitrate reduction		–	–	–	–	–	–
Carbohydrate utilization	Glucose	+	+	+	+	+	ND
	Fructose	–	–	+	–	+	–
	Lactose	–	+	+	+	+	–
	Raffinose	–	–	–	–	–	+
	Arabinose	–	–	+	+	ND	
	Melobiose	–	+	+	+	+	
	Cellobiose	–	–	–	ND	–	ND
	Rhamnose	–	–	–	–	–	–
Hydrolysis of	Casein	–	–	–	–	+	+
	Gelatin	–	–	–	–	–	+

Strain 1, *Muricauda* sp.YUAB-SO-11; Strain 2; *M. olearia* YUAB-SO-45; Strain 3, *M. aquimarina* JCM 11811^T, Strain 4, *M. olearia* JCM 15563^T, Strain 5, *M. lutaonensis,* KCTC 22339^T, Strain 6, *M. lutimaris* KCTC 22173^T, Data from Arun *et al.,* 2010, Hwang *et al.,* 2009; Yoon *et al*., 2005 and this study. All the strains are negative for agar and starch hydrolysis. They do not produce urease and H_2S. All strains require NaCl for growth.
+, Positive; –, Negative; ND, no data available; w, weakly positive.

Table II
Total biomass and zeaxanthin yield produced by *Muricauda* strains cultured in marine broth at 32°C or for *M. lutaonensis* at 42°C at 150 rpm.

Parameters	Growth time (h)	CDW (g/mL)	Zeaxanthin (mg/L)
Muricauda sp. YUAB-SO-11	48	2.6 ± 0.2	3.14 ± 0.2
Muricauda olearia YUAB-SO-45	72	2.1 ± 0.2	2.16 ± 0.1
Muricauda aquimarina (JCM 11811^T)	72	2.3 ± 0.2	3.5 ± 0.1
Muricauda flavescens (JCM 11812^T)	72	2.9 ± 0.1	4.4 ± 0.2
Muricauda lutaonensis (KCTC 22339^T)	72	1.9 ± 0.1	3.1 ± 0.1
Muricauda lutimaris (KCTC 22173^T)	72	2.3 ± 0.1	0.7 ± 0.1

Further investigation of the pigment by HPLC analysis resolved the zeaxanthin with a prominent peak (RT, 10.8) comparable to standard zeaxanthin. This fraction was subjected to mass analysis which exhibited a parent ion at *m*/*z* 569 and collusion-induced disassociation fragments of *m*/*z* 551 and 419 which were identical to that of standard all-*trans*-zeaxanthin (Fig. 2). The compounds corresponding to peak-2 (RT, 13.87) and peak-3 (RT, 14.65) were predicted to be other minor carotenoids respectively. The zeaxanthin produced by *M. flavescens* JCM 11812^T (4.4 ± 0.2 mg L^{-1}) was highest while by *M. lutimaris* KCTC 22173^T was the lowest (0.7 ± 0.1 mg L^{-1}) (Table II). These results indicate that zeaxanthin is the major pigment in the genus *Muricauda*.

Zeaxanthin biosynthesis occurs *via* the mevalonate pathway (MVA) or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway by enzymatic reaction of β-carotene hydroxylase (*CrtZ*) which modifies the β-ring (β-carotene) to zeaxanthin (Misawa 2011). Whole genome sequencing of the strain *M. rustringenesis* DSM 13258^T (Huntemann *et al*., 2012) showed the presence of *CrtZ* gene. Accumulation of high concentration of zeaxanthin in marine microorganisms including strains

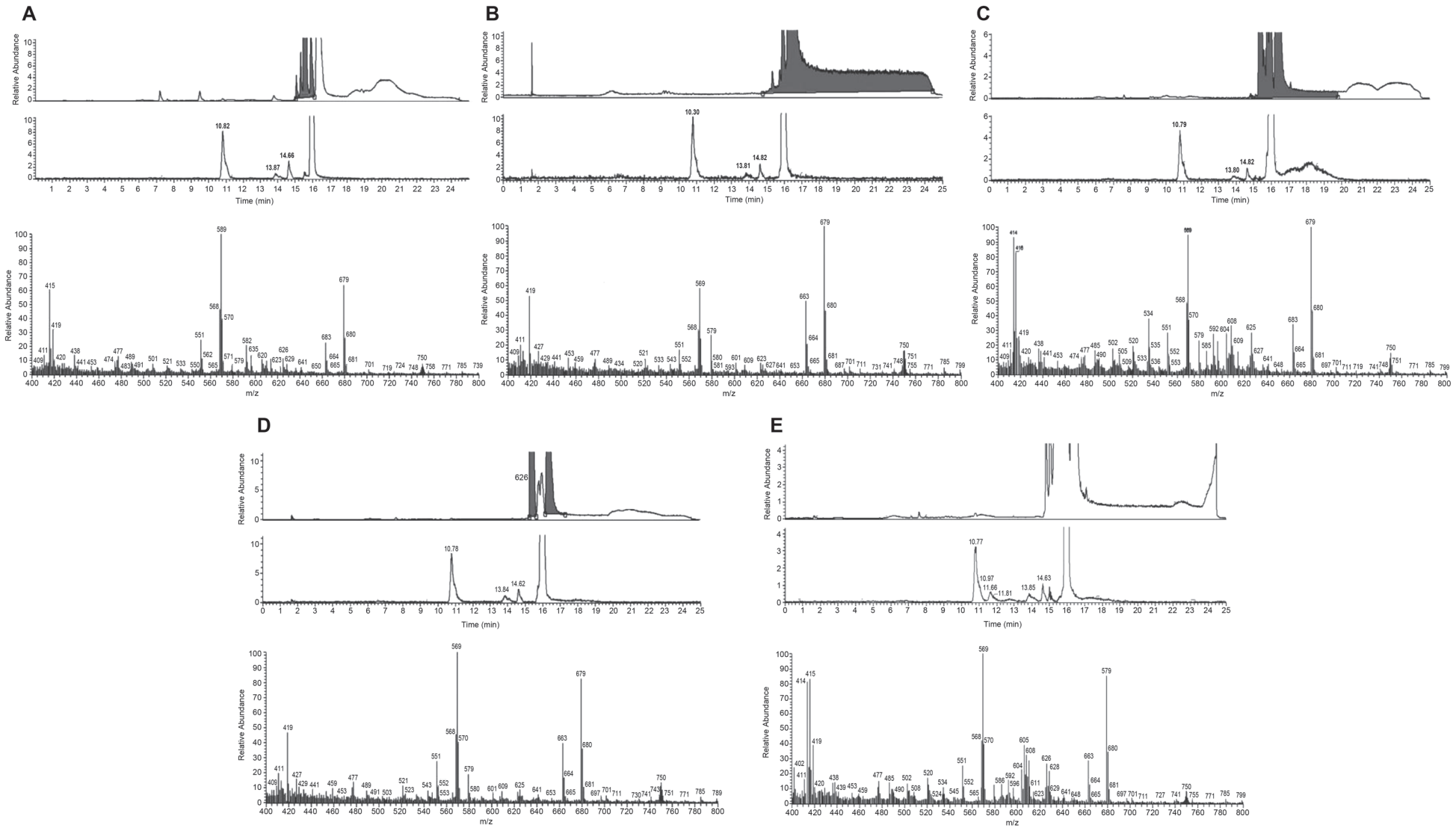

Fig. 2. LC-MS/MS analysis of the crude ethanol extract of five *Muricauda* strains (**A**, *Muricauda* sp. YUAB-SO-11; **B**, *M. olearia* YUAB-SO-45; **C**, *M. flavescens* JCM 11812^{T}; **D**, *M. aquimarina* JCM 11811^{T}; **E**, *M. lutimaris* KCTC 22173^{T}). Chromatogram shows a peak at 10.92 minutes compared with the zeaxanthin standard. (B) MS/MS spectrum of carotenoid from all the strains of *Muricauda* showed a parent peak at *m*/*z* 569, and subsequent fragments at *m*/*z* 551 and *m*/*z* 416.

from genus *Muricauda* might have an evolutionary role in survival, in membrane stabilization, UV tolerance, and as antioxidant protecting cell from oxidative stress (Asker *et al*., 2012). Combining the information from this study and available published data on zeaxanthin, particularly from microbial source, it is evident that zeaxanthin production is an important phylogenetic trait of this genus. Increasing demand for zeaxanthin, particularly from microbial source has gained significant interest due to its identical stereochemistry to natural zeaxanthin which has lead researchers to identify potential zeaxanthin producing strains. *Sphingobacterium multivorum* produced high zeaxanthin yield (Bhosale *et al*., 2004), whereas *Muricauda* species produce zeaxanthin as a single predominant carotenoid in higher quantities indicating strains from this genus likely be potential candidates for large scale production of zeaxanthin. Comparison of carotenoid distribution in *Muricauda* strains with their 16S rRNA gene phylogeny indicates that the carotenoid distribution and composition is highly conserved between the species. In the genus *Muricauda*, the uniform distribution of zeaxanthin strongly suggests that the carotenoid biosynthetic pathway may be less evolutionary elastic resulting in only certain structural types existing without giving further scope for the diversification of carotenoids within the genus (Klassen 2009; 2010).

The result of this study further enhances our current limited knowledge regarding the distribution and diversity of carotenoid in family *Flavobacteriaceae*. There is further scope for investigating the evolution of carotenoids in these species within the concept of biotechnology and exploiting their metabolic diversity.

This research work was kindly supported by a grant from Board Research of Nuclear Science BRNS 28/34/2009.

Literature

Arun A.B., W.M. Chen, W.A. Lai, J.H. Chao, P.D. Rekha, F.T. Shen, S. Singh and C.C. Young. 2009. *Muricauda lutaonensis* sp. nov., a moderate thermophile isolated from a coastal hot spring. *Int. J. Syst. Evol. Microbiol.* 59: 2738–2742.

Asker D., T.S. Awad, T. Beppu and K. Ueda. 2012. Novel zeaxanthin-producing bacteria isolated from radioactive hot spring water. *Methods Mol. Biol.* 892: 99–131.

Asker D., T. Beppu and K. Ueda. 2007(a). *Mesoflavibacter zeaxanthinifaciens* gen. nov., sp. nov., a novel zeaxanthin-producing marine bacterium of the family *Flavobacteriaceae*. *Syst. Appl. Microbiol.* 30: 291–296.

Asker D., T. Beppu and K. Ueda. 2007(b). *Zeaxanthinibacter enoshimensis* gen. nov., sp. nov., a novel zeaxanthin-producing marine bacterium of the family *Flavobacteriaceae*, isolated from seawater off Enoshima Island, Japan. *Int. J. Syst. Evol. Microbiol.* 57: 837–843.

Asker D., T. Beppu and K. Ueda. 2007(c). *Sphingomonas jaspsi* sp. nov., a novel carotenoid-producing bacterium isolated from Misasa, Tottori, Japan. *Int. J. Syst. Evol. Microbiol.* 57: 1435–1441.

Bhosale P. and P.S. Bernstein. 2004. Beta-carotene production by *Flavobacterium multivorum* in the presence of inorganic salts and urea. *J. Ind. Microbiol. Biotechnol*. 31: 565–571.

Bruns A., M. Rohde and L. Berthe-Corti. 2001. *Muricauda ruestringensis* gen. nov., sp. nov., a facultatively anaerobic, appendaged bacterium from German north sea intertidal sediment. *Int. J. Syst. Evol. Microbiol*. 51: 1997–2006.

Hameed A., A.B. Arun, H.P. Ho, C.M. Chang, P.D. Rekha, M.R. Lee, S. Singh and C.C. Young. 2011. Supercritical carbon dioxide micronization of zeaxanthin from moderately thermophilic bacteria *Muricauda lutaonensis* CC-HSB-11^{T}. *J. Agric. Food. Chem.* 59: 4119–4124.

Hameed A., M. Shahina, S.Y. Lin, K.R. Sridhar, L.S. Young, M.R. Lee, W.M. Chen, J.H. Chou and C.C. Young. 2012. *Siansivirga zeaxanthinifaciens* gen. nov., sp. nov., a novel zeaxanthin-producing member of the family *Flavobacteriaceae* isolated from coastal seawater of Taiwan. *FEMS Microbiol. Lett*. 333: 37–45.

Huntemann M., H. Teshima, A. Lapidus, M. Nolan, S. Lucas, N. Hammon, S. Deshpande, J.F. Cheng, R. Tapia, L.A. Goodwin and others. 2012. Complete genome sequence of the facultatively anaerobic, appendaged bacterium *Muricauda ruestringensis* type strain B1^{T}. *Stand. Genomic. Sci.* 6: 185–193.

Hwang C.Y., M.H. Kim, G.D. Bae, G.I. Zhang, Y.H. Kim and B.C. Cho. 2009. *Muricauda olearia* sp. nov., isolated from crude-oil-contaminated seawater, and emended description of the genus *Muricauda*. *Int. J. Syst. Evol. Microbiol*. 59: 1856–1861.

Kämpfer P., U. Dreyer, A. Neef, W. Dott and H.J. Busse. 2003. *Chryseobacterium defluvii* sp. nov., isolated from wastewater. *Int. J. Syst. Evol. Microbiol*. 53: 93–97.

Klassen J.L. 2009 Pathway evolution by horizontal transfer and positive selection is accommodated by relaxed negative selection upon upstream pathway genes in purple bacterial carotenoid biosynthesis. *J. Bacteriol.* 191: 7500–7508.

Klassen J.L. 2010. Phylogenetic and evolutionary patterns in microbial carotenoid biosynthesis are revealed by comparative genomics. *PLoS ONE* 5: 11257.

Krinsky N.I., J.T. Landrum and R.A. Bone. 2003. Biological mechanisms of the protective role of lutein and zeaxanthin in the eye. *Annu. Rev. Nutr.* 23: 171–201.

Lee S.Y., S. Park, T.K. Oh and J.H. Yoon. 2012. *Muricauda beolgyonensis* sp. nov., isolated from a tidal flat. *Int. J. Syst. Evol. Microbiol*. 62: 1134–1139.

Misawa N. 2011. Carotenoid β-ring hydroxylase and ketolase from marine bacteria promiscuous enzymes for synthesizing functional xanthophylls. *Mar. Drugs* 9: 757–771.

Sanusi R.A. and A.E. Adebiyi. 2009. Beta carotene content of commonly consumed foods and soups in Nigeria. *Pak. J. Nutr.* 8: 1512–1516.