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Isolation and Characterization of Marine Pigmented Bacteria from Norwegian Coastal Waters and Screening for Carotenoids with UVA-Blue Light Absorbing Properties§

Marit H. Stafsnes[1], Kjell D Josefsen[2], Geir Kildahl-Andersen[3#], Svein Valla[1], Trond E. Ellingsen[2], and Per Bruheim[1,2*]

[1]Department of Biotechnology, Norwegian University of Science and Technology Sem Sælands vei 6/8, NO-7491 Trondheim, Norway
[2]SINTEF Materials and Chemistry, Department of Biotechnology, Sem Sælands vei 2a, NO-7465 Trondheim, Norway
[3]Department of Chemistry, Norwegian University of Science and Technology Sem Sælands vei 6/8, NO-7491 Trondheim, Norway
[#]Present address: Department of Chemistry, University of Oslo, P.O. Box 1033, Blindern, N-0315 Oslo, Norway

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Microbial culture collections are important resources for isolation of natural compounds with novel properties. In this study, a culture collection of around 1,500 pigmented heterotrophic bacteria was established. The bacteria were isolated from the sea surface microlayer at different sampling sites along the mid-part of the Norwegian coast. The bacterial isolates produced pigments of various coloration (e.g. golden, yellow, red, pink and orange). Methanol extracts of sixteen isolates were characterized with LC-Diodearray-TOF mass spectrometry analysis. The number of pigments per isolate varied considerably, and a tentative identification of the pigments was performed based on UV-absorbance profile and molecular formula assignation based on the accurate mass determination. The LC-MS analyses revealed that most of the pigments probably were carotenoids. Furthermore, we developed a high throughput LC-MS method for characterization and screening of a larger sub-fraction (300 isolates) of the culture collection. The aim was to screen and identify bacterial isolates producing carotenoids that absorb light in the UVA-Blue light. Six of the bacterial strains were selected for detailed investigation, including 16s rRNA sequencing, preparative HPLC for purification of major carotenoids and subsequent structural elucidation with NMR. Among the identified carotenoids were zeaxanthin, nostoxanthin and sarcinaxanthin, some with novel glycosylation patterns.

***Keywords*:** bacterial culture collection, high throughput screening, LC-MS, pigments, carotenoids

Pigmentation is widespread among bacteria and pigments found in marine heterotrophic bacteria comprise carotenoid, flexirubin, xanthomonadine, and prodigiosin (Reichenbach *et al.*, 1980; Kim *et al.*, 2007). The carotenoids are considered to be the main and most abundant pigment group. Carotenoids are produced by a wide variety of organisms, from non-phototrophic prokaryotes to higher plants, with over 700 different structures identified to date. They appear either yellow, orange, or red and the biosynthesis of the carbon skeleton is based on condensation of isoprenyl units. A large variety of structures are obtained through various modifications (oxidation, hydroxylation, etc) of the carbon backbone. Because of their color and biological properties (i.e. antioxidant), carotenoids are used commercially as food colorants, animal feed supplements and, more recently, as nutraceuticals for cosmetic and pharmaceutical purposes. Currently, commercial carotenoid production is mostly based on extraction from plant tissues or chemical synthesis. However, owing to a growing worldwide market for these compounds microbial production has great potential in terms of both the efficiency of production and the diversity of carotenoid structures (de Haan *et al.*, 1991; Vandammen, 1992; Vázquez and Martin, 1997; Buzzini and Martin, 1999; Buzzini, 2001; Frengova *et al.*, 2003).

Many of the heterotrophic bacteria that synthesize carotenoids have been isolated from coastal and oceanic waters (Yurkov and Beatty, 1998; Du *et al.*, 2006). The widespread occurrence of carotenoids in non-phototrophic bacteria suggests that their presence 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 protection from photo-oxidative damage and in absorption of visible light (Luckner, 1990; Britton *et al.*, 1995). Culture collections of pigmented bacteria can be a valuable resource for isolating new carotenoids with interesting properties, especially if the collection displays a large diversity in carotenoid structures. An advantage with bacterial culture collections is that they are easy to screen using high throughput technologies, i.e. cultivation in well-plate formats with robotic extraction of carotenoids followed by rapid resolution LC-MS analysis.

In this paper, we describe our recently established extensive collection of pigmented heterotrophic bacteria isolated from coastal waters of Mid-Norway. Through two different LC-MS screens we found a large diversity in carotenoid production among the different isolates. Finally, we show how the collection can be used to screen for carotenoids with particular

* For correspondence. E-mail: Per.Bruheim@biotech.ntnu.no;
Tel: +47-7359-3321; Fax: +47-7359-1283
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interesting properties, i.e. UVA-Blue light range (350-450 nm) absorbing carotenoids. Such carotenoids may be used as additives in sun protection products as most sun screen products show poor protection against UVA-Blue light, even though light of these wavelengths are known to damage the epidermic layer. Six isolates producing UVA-Blue absorbing carotenoids were selected for a detailed investigation including purification and structural identification of several major carotenoids.

Materials and Methods

Sampling

Marine samples were collected from surface water at 17 different sites along the coast of Mid-Norway. Sampling locations and conditions are shown in Supplementary data Table 1 and a map of the sampling locations is given in Supplementary data Fig. 1. The sea surface microlayer was sampled with Teflon plates as described earlier (Kjelleberg *et al.*, 1979). The plates were immersed in water and gently lifted horizontally through the water surface. The adhering surface film was scraped off using a rubber edge and collected in a funnel closed with a rubber stopper in the bottom. The procedure was repeated until a total volume of 30-50 ml was collected. The thickness of the water films adhering to the Teflon plates were calculated from the plate surface area and the average volume obtained per sampling with the plate. The samples were stored on ice until further processing which was always carried out within 24 h. The salinity was determined as described by (Müller, 1999).

Isolation of pigmented colonies

The samples were aseptically diluted in a ten-fold series in sterile media (composition listed below) up to 10^{-5}. From each dilution 5×0.25 ml were spread on 14 cm agar plates, which were incubated at 18°C for 2 months or at 25°C for 1 month.

The low-nutrient artificial seawater agar medium, with a salinity of approx. 25‰ and pH 7.6, had the following composition (g/L): NaCl, 17.1; $MgCl_2 \cdot 6H_2O$, 7.8; Na_2SO_4, 2.9; $CaCl_2 \cdot 2H_2O$, 1.1; KCl, 0.50; KBr, 0.12; washed agar [prepared by washing 50 g agar (Agar No. 1, Oxoid)] on a filter paper (520B ½ Folded filters Φ 185 mm, Schleicher & Schuell) with 5×0.5 L distilled water, and then freeze-drying the agar), 15, and (mg/L): $NaHCO_3$, 136, peptone (Oxoid), 100; yeast extract (Oxoid), 20; H_3BO_3, 17.9; $SrCl_2$, 10.2; NH_4NO_3, 8; NaF, 2.1; KH_2PO_4, 1.8. In addition were added 20 µl vitamin solution, and 40, 4, and 2 µl of trace mineral solution (TMS) 1, 2, and 3, respectively. The vitamin solution contained (mg/L): *myo*-inositol; 300; thiamineHCl, 100; niacinamide, 100; *d*-pantothenic acid, 100; folic acid, 100; riboflavin, 50; pyridoxine HCl, 50; lipoic acid, 50; vitamin B_{12}, 5, and *d*-biotin, 5. The three trace mineral solutions contained : TMS 1 (per L): $FeSO_4 \cdot 7H_2O$, 5.0 g; $CuSO_4 \cdot 5H_2O$, 0.39 g; $ZnSO_4 \cdot 7H_2O$, 0.44 g; $MnSO_4 \cdot H_2O$, 0.15 g; $Na_2MoO_4 \cdot 2H_2O$, 10 mg; $CoCl_2 \cdot 6H_2O$, 20 mg, and conc. HCl, 50 ml. TMS 2 (per L): $K_2B4O_7 \cdot 4H_2O$, 5.7 g; $Na_2SiO_3 \cdot 9H_2O$, 2.0 g; NaF, 0.45 g; $KAl(SO_4)_2 \cdot 12\ H_2O$, 0.18 g; $NaIO_3$, 48 mg; $SnCl_2 \cdot\ 2H_2O$, 11 mg, and conc. HCl, 50 ml. TMS 3 (per L): $NiCl_2 \cdot 6H_2O$, 0.35 g; $Na_2SeO_3 \cdot 5H_2O$, 0.10 g; V_2O_5, 18 mg; $K_2Cr_2O_7$, 14 mg; $Na_2WO_4 \cdot 2H_2O$, 4 mg, and conc. HCl, 50 ml. To prevent growth of fungi, 250 µl nystatin solution (2.9 g/L in DMSO) (Sigma, J.T. Baker) were added to the agar plates the day before the plates were used. Chemicals were obtained from Oxoid and Sigma unless otherwise specified. From now on general precautions for work with carotenoids were taken (Schiedt and Liaaen-Jensen, 1995).

All yellow to red colored colonies from two dilutions (all parallels) containing between one and 400 colonies were picked and inoculated in deep well plates (96-well plates) containing 0.6 ml Yeast Peptone Salt medium (YPS) with the following composition (g/L): peptone, 5; yeast extract, 1; NaCl, 15; sea salt (Tropical Marine®, Dr. Biener GmbH, Germany), 15; and K_2HPO_4, 0.01. Phosphate was added after heat sterilization and finally pH was adjusted to 7.6. The deep 96-well plates were incubated with shaking (800 rpm) at 25°C for 2-3 weeks or 18°C for 3-4 weeks (same incubation temperature as agar plates), added glycerol to a final concentration of 20% and thereafter distributed in aliquots of 0.2 ml to five standard 96-well plates. The well plates were sealed with plastic film and stored at -80°C.

Cultivation

Cultivations for production of biomass for characterization of the culture collection were performed in 96-well plates, shake flasks, and fermentors. The cultures were inoculated from the frozen culture collection and an inoculum stage was used for the shake flask and the fermentation, while the micro-well cultivations were inoculate directly with an aliquot (10 µl) from a thawed collection plate. Deep 96-well plates with 0.6 ml YPS medium were used for micro-well cultivations. At the end of the incubation the plates were centrifuged (650×g, 15 min) and washed twice with phosphate buffered saline and freeze dried.

Shake flask cultivations were performed in 500 ml shake flasks filled with 100 ml YPS medium. They were incubated for 4-5 days at 25°C in a Minitron shaking incubator (orbital 25 mm) operated at 200 rpm. Aliquots of 50 ml were harvested by centrifugation at 7,000 rpm (13,900×g), washed with 10 ml of phosphate buffered saline, and recentrifuged. The cell pellets were freeze-dried and stored at -20°C prior to extraction of pigments.

A larger amount of cell mass was necessary for those isolates that underwent detailed analysis, and fermentors (Applicon) with operating volume of 1 L were used to produce the cell mass. Shake flask cultures (50 ml) in late growth phase were used to inoculate the fermentors. The final concentration of the basic medium was (g/L): glucose, 22; peptone, 10; yeast extract, 3; NaCl, 15; Tropical marine sea salt, 15; K_2HPO_4, 2, and per L 5 ml trace mineral solution (g/L: $FeSO_4$, 50.0; $CuSO_4 \cdot 5H_2O$, 3.9; $ZnSO_4 \cdot 7H_2O$, 4.4; $MnSO_4 \cdot H_2O$, 1.5; $Na_2MoO_4 \cdot 2H_2O$, 0.1; $CoCl_4 \cdot 6H_2O$, 0.2; concentrated HCl, 50 ml) was added. The pH was maintained at 7.8 by automatically adding 2 M NaOH during cultivation. Dissolved oxygen (DO) was monitored and not allowed below 35% of saturation, this was adjusted by increasing the stirring rate from 300 to 1,000 rpm while the air flow was kept constant at 0.2 L/min. CO_2 production was continuously monitored during the fermentations with a Rosemount Binos 100 CO_2 analyzer. The temperature was maintained at 25°C and Clerol anti-foam agent was added when necessary to prevent foaming. After one day of cultivation, 2 ml vitamin solution (same composition as for low nutrient agar), 6.8 g NH_4NO_3, and 5 g yeast extract were added to the fermentors.

Extraction of pigment

The washed cell pellet was freeze dried to avoid degradation of carotenoids during storage at -20°C and to facilitate extraction with organic solvents. Lyophilized well plates were extracted with 300 µl methanol and incubated for 3 h at room temperature (RT) on a shaker plate. After centrifugation, 200 µl from each well was transferred to HPLC vials. The lyophilized cell pellet from shake flasks and fermentors were added 1 ml methanol and mechanically mixed and vortexed for 10 sec to ensure even dispersal of the biomass. It was then transferred to a 50 ml Falcon tube and added another 9 ml MeOH. The tubes were flushed with N_2 and wrapped in aluminum

foil to prevent light mediated oxidation of the extracted carotenoids, and then incubated for 2 h on a rotary shaker (50 rpm) at RT. The suspensions were centrifuged (3,200×g, 10 min, 4°C), methanol phase removed and concentrated to a final volume of ~1 ml by N_2 evaporation. This extraction protocol did not work satisfactory for some of the isolates and a cell disintegration step was included. Between 10 and 50 mg of lyophilized cells were weighed exactly into 2 ml reaction tubes (Eppendorf, Germany) and 1,200 µl PBS was added. Furthermore, 200 µl of lysozyme solution (2,000 U/µl) (Sigma-Aldrich) was added. Enzyme solutions were prepared every day in PBS (pH 7.4). Tubes were vortexed for 30 sec and the suspension incubated for 1 h at 37°C on a horizontal shaker (250 rpm, orbital 2.5 mm). After this pre-treatment the standard protocol for pigment extraction was followed.

Analytical LC-MS and preparative HPLC

High throughput LC-MS analyses were performed on an Agilent Single Quadrupol SL mass spectrometer equipped with an Agilent 1100 series HPLC system. The LC system was equipped with a diode array detector (DAD) that recorded UV/VIS spectra in the range from 200-650 nm. Mobile phases were methanol-water (80:20) in channel A and dichloromethane in channel B. The carotenoids were eluted with an increasing percentage of mobile phase B using the following gradient: 0% B from 0-0.5 min, then a linear gradient to 70% B after 4 min. The column was re-equilibrated with a 3 min post run giving a total run time of 7 min. A Zorbax rapid resolution cartridge RP C_{18} column with dimension 2.1×30 mm was used for the analyses. Column flow was kept at 0.5 ml/min and 10 µl extract was injected for each run. Analytes were ionized using a chemical ionization source with settings 325°C dry temperature, 350°C vaporizer temperature, 50 psi nebulizer pressure and 5.0 L/min dry gas. The MS was operated in single ion monitoring (SIM) mode for 90% of cycle time and in scan mode for 10% of the cycle time. 21 carotenoid masses ($M+H^+$) were included in the SIM list (Supplementary data Table 2). ß-Carotene (Sigma) and astaxanthin (Sigma) were used as external standards to check that retention times remained constant during the runs. Samples and standards were filtered through a syringe polypropylene filter (0.2 µm, Pall Gelman) and stored in amber glass vessels at -80°C under N_2 atmosphere if not analyzed immediately.

High mass accuracy analyses were performed on an Agilent Time of flight (TOF) mass spectrometer equipped with an Agilent 1100 series HPLC system equipped with a diode array detector. Mobile phases and ion source conditions were the same as for the high throughput analysis. Reference ions for calibration of mass axis were tee-ed into the mobile phase after the column separation. This enables routine accurate mass determination with better than 3 ppm accuracy. Chromatographic separation was performed with an Ace RP C_{18} 4.6×150 mm column operated with flow of 1 ml/min. The following gradient was used: 0% B for 0-2 min, 0-30% B from 2 to 12 min, 30-60% B from 12-18 min.

Preparative HPLC was performed on an Agilent Preparative HPLC system equipped with two preparative HPLC pumps, a preparative autosampler and a preparative fraction collector. Mobile phases were methanol in channel 1 and dichloromethane in channel 2. The mobile phase gradient had to be optimized for each of the different extracts. 5 ml samples were injected at a flow rate of 85 ml/min to a Zorbax RP C18 50×250 mm preparative LC column. On line MS analysis was performed by splitting the flow 1:200 after the column using an Agilent LC flow splitter and a make-up flow of 1 ml methanol/min to carry the analytes to the MS with less than 15 sec delay. The diode array detector was used to trigger fraction collection.

Table 1. Accurate mass determination and molecular formula calculation of the six most prominent carotenoids in the sample presented in Fig. 1. Rt is retention time in minutes and points to the six spectra in Fig. 1B

Rt (min)	Measured mass	Molecular formula	Theoretical mass	ppm error
10.7	567.4192	$C_{40}H_{54}O_2$	567.4196	0.8
11.4	569.4335	$C_{40}H_{56}O_2$	569.4353	3.1
11.8	567.4187	$C_{40}H_{54}O_2$	567.4196	1.6
12.3	567.4192	$C_{40}H_{54}O_2$	567.4196	0.8
12.7	569.4348	$C_{40}H_{56}O_2$	569.4353	0.8
14.3	551.4247	$C_{40}H_{54}O$	551.4231	2.9

NMR

All NMR measurements were performed using a Bruker Avance 600 MHz spectrometer, equipped with a 5 mm CryoProbe™ (TCI). Standard ^{1}H NMR and 2D ^{1}H,^{1}H COSY experiments were performed. Most of the samples could be dissolved in $CDCl_3$, except for samples containing glycosylated carotenoids, for which either CD_3OD, pyridine-d_5 or a mixture of CD_3OD and acetone-d_6 had to be employed.

Results and Discussion

Establishment of the collection of heterotrophic pigmented marine bacteria and extraction of pigments

Preliminary experiments for establishing the culture collection were conducted to develop the sampling technology. This included sampling at the surface, at 20 cm and at 10 m depth. There were more cultivable bacteria in the surface layer, and there was also a relatively higher proportion of pigmented bacteria at the surface, probably due to the photoprotecting properties of pigments as also indicated by others (Du *et al.*, 2006). It was therefore decided that the sampling for the culture collection of heterotrophic pigmented bacteria should continue only from the surface layer of the water column. It was also observed a higher colony count on plates with a low nutrient artificial seawater agar than on more nutrient rich agar plates, possibly because very few colonies grew large on the low nutrient agar during the incubation period. Thus, slow-growing bacteria that would otherwise be overgrown by rapidly expanding colonies were allowed more time to develop into visible colonies on the low nutrient agar medium.

More than 1500 pigmented bacterial strains were isolated from a total of 20 samples collected from 17 different sampling sites along the coastal surface water in Mid-Norway between autumn 2004 and summer 2005. Sampling locations are shown on map in Supplementary data Fig. 1 while Table 1 contains information about thickness of the surface micro layer, salinity and sea temperature at time of sampling. The thickness of the surface layer was calculated by measuring the average volume of the liquid film adhering to the Teflon plate. Originally over 3,500 pigmented isolates were picked form the first agar isolation step. But approximately 2,000 of the isolates did not grow or did not produce any pigments during second step re-cultivation in liquid culture. Hence, they were discarded from the collection. One must take into account that the high

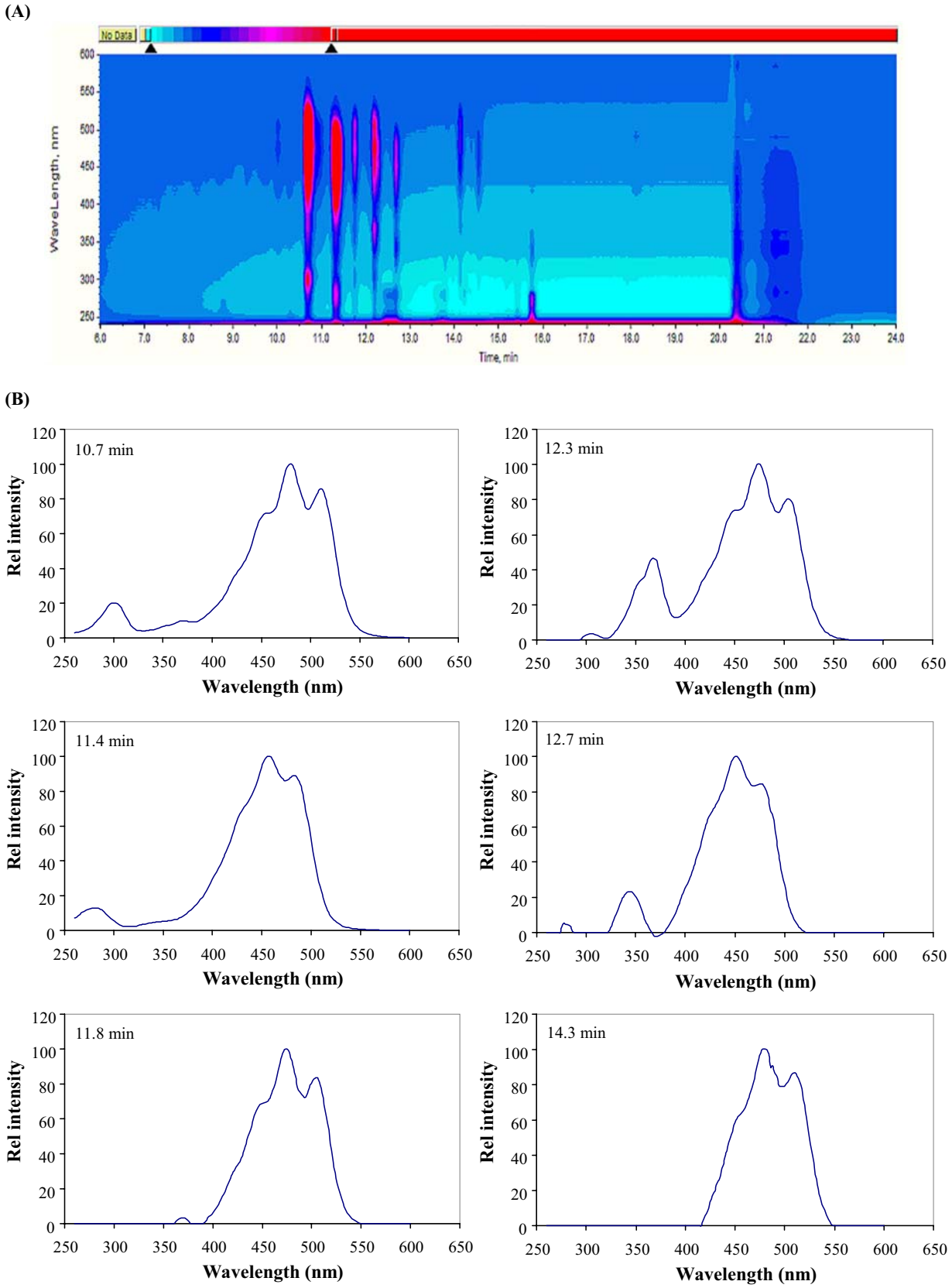

Fig. 1. Diodearray isoabsorbance plot of a methanol extract from a representative bacterial isolate from the culture collection. The UV-VIS range from 250 nm to 600 nm is plotted vs the zoomed in chromatogramic period from 6 min to 24 min (A). UV/ VIS-scan of the three major and three minor pigments in the extract (B). The time indicated in the scans reflects the retention time from the isoabsorbance plot in Fig. 1A.

diversity in the marine environment is hard to reproduce in the laboratory. In seawater the culturability is reported as 0.001-0.1% as the cultivation method selects for certain organisms (Amann *et al.*, 1995).

Carotenoids are lipophilic and practically insoluble in water, but readily soluble in organic solvents such as acetone, alcohols, ethyl ether, chloroform, DMSO, and ethyl acetate. After initial tests on ten different isolates we found that pure methanol gave the best overall extraction efficiency with this heterogeneous collection of bacterial isolates. The extraction was not complete for all isolates since pigments clearly remained in the pellet after extraction. However, the characterization of selected isolates in the culture collection was only qualitative and a complete extraction was therefore not required. A pre-treatment with lysozyme was added to the extraction procedure for the isolates that were selected for detailed analysis including purification and structural elucidation of major carotenoids.

Initial pigment screen – LC-TOF-MS analysis

Sixteen isolates were randomly chosen from the culture collection for a detailed LC-DAD-TOF MS analysis. This initial analysis would give important information for designing the analysis protocol for screening of a high number of isolates later. The 16 isolates were grown in shake flasks, and the harvested cell pellets freeze-dried prior to methanol extraction and subsequent LC-DAD-TOF MS analysis. The objective was to use the combination of diode array detection (DAD) and high mass accuracy determination to tentatively identify the individual carotenoids in each of the extracts. The number of carotenoids in the individual extracts varied but most of the 16 extracts contained 3-5 major carotenoids and several minor carotenoids (data not shown). Figure 1A shows a diode array plot of a representative extract. This extract contains three major and at least three minor carotenoids. The UV/VIS scans for the six largest peaks are shown in Fig. 1B. They display some difference in absorbance profile, but several peaks also share strong similarities. The TOF MS was used to assign accurate mass to each of these six peaks (Table 1). All masses were within a 3 ppm error range of known carotenoid masses found by searching the Dictionary of Natural Products (DNP) database (http://www.chemnetbase.com). DNP contain information about accurate mass, molecular formula, absorbance profile etc. for over six hundred carotenoids. Since many carotenoids have the same molecular formula accurate mass is not enough to provide a unique identification of the detected carotenoids (stereochemistry not involved in this discussion), but combined with the LC retention time index and UV scan it is possible to discriminate between different carotenoids in the various extracts. The UV scan can sometimes be a unique identifier for specific carotenoids and both DNP and the recently revised Handbook of Carotenoids contain extensive information about UV absorbance profile of known carotenoids (Liaaen-Jensen *et al.*, 2004). However, this information could only be used tentatively in this investigation because peak maximum changes with mobile phase composition. The data in DNP and in the Handbook are recorded in pure solvent systems and even though the profile matches, the peak maximum can be shifted during the gradient elution of the LC-MS run. The m/z 567.419 occurs three times in the chromatogram. This corresponds to the molecular formula $C_{40}H_{54}O_2$ for which there are 12 entries in DNP. The spectra of the three carotenoids show close resemblance varying only in the 300-400 nm range. These three carotenoids could be isomers i.e. *cis-trans* or Z/E of the same carotenoid. In Nature carotenoids exist primarily in the thermodynamically more stable all-*trans* conformation. *Trans–cis* conversion is probably formed during extraction and sample processing (Molnar and Szabolcs, 1993; Britton *et al.*, 1998).

The culture collection was established by random picking of colored colonies appearing during the first enrichment step on low nutrient agar and the same species and strain is likely to be picked more than once. Assuming that isolates with matching diode array plot and accurate mass represent the same bacterial strain this suggest that the sixteen randomly chosen isolates represent eight different bacterial strains. Four strains occurred only once, one two times, two three times, and one four times. To visualize the variation among the isolates diode array plot of four of these unique isolates are shown in Fig. 2. Clearly, the isolates produce both hydrophobic caroteoids (eluting late in the chromatogram) and more hydrophilic carotenoids (eluting rapidly in the chromatogram). All together, this analysis of only 16 isolates indicates a large caroteoid diversity among the isolates in the culture collection.

We obtained several masses from the LC-TOF analysis with carotenoid-like absorption spectra that did not give any hits in DNP and these are all potential novel and previously undiscovered pigments. Pigments with masses higher than 850 may be glycosylated carotenoids, i.e. carotenoids linked to a sugar by a glycosidic or an ester link. The first carotenoid glycoside was isolated from saffron and described in 1818 (Aschoff, 1818). The utility of carotenoid glycosyl esters as colorants for textiles and food, as antioxidant quenchers for free radicals and as antitumor agents has been reported (Dembitsky, 2005). So far few glycosilated carotenoids have been reported compared to the variety of carotenoids reported although it is assumed that a substantial amount of bacterial carotenoids often are glycosylated (Britton, 1998; Krubasik *et al.*, 2001; Takaichi *et al.*, 2003). Other natural pigments that do not belong to the carotenoid family but have a similar terpenoid conjugated backbone structure and therefore also similar absorbance spectra are also described in the literature. These are flexirubin, xanthomonadin, xanthomonasin a, xanthomonasin b and prodigiosin (Reichenbach *et al.*, 1980; Rajagopal *et al.*, 1997; Kim *et al.*, 2007). No further attempts were made to identify these unknown compounds.

High throughput LC-MS screen of 260 isolates

The next step was to characterize a higher number of isolates in the collection. The LC-TOF-MS analysis takes almost 30 min per run which is too long for screening purposes. We therefore established a 7 min LC method using a rapid resolution HPLC column. Our goal for the screening was to characterize the isolates for diversity and abundance of carotenoids. In addition, we were particularly interested in carotenoids with a low λ_{max} in their UV/VIS absorbance profile. The chromatographic resolution may decrease with a shorter run period and carotenoids that are separated in a long analysis run can co-elute in a shorter run. However, screening and initial characterization of isolates focus on the major

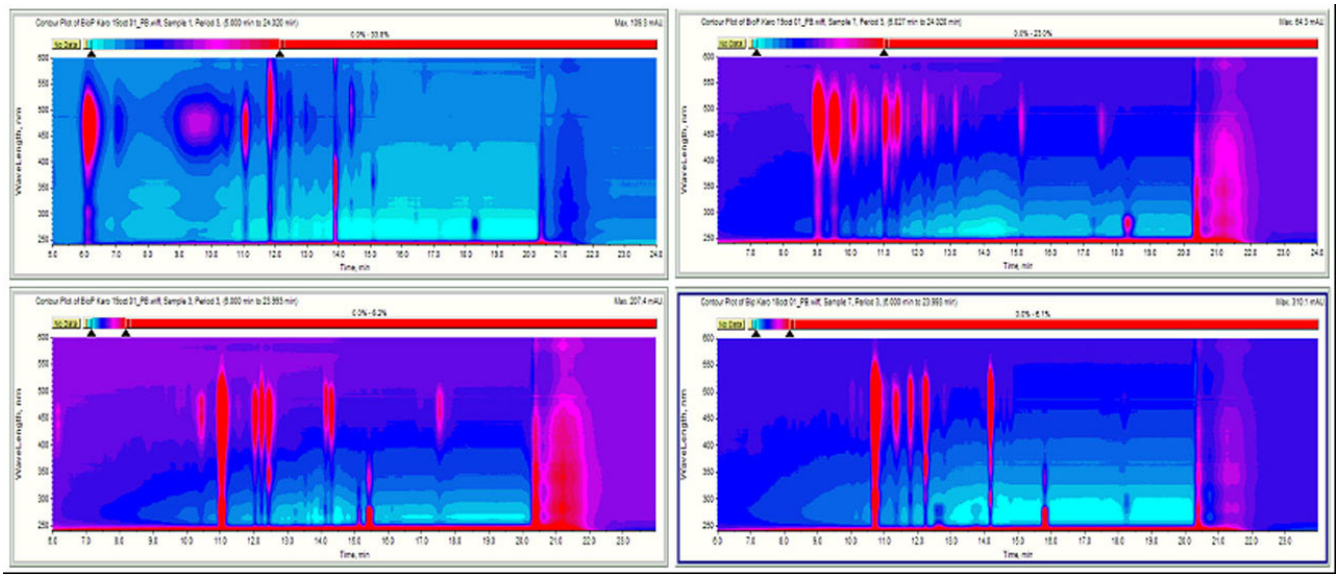

Fig. 2. Diodearray isoabsorbance plot of four unique bacterial isolates from the culture collection. The isolates were chosen to visualize the large variation among the isolates.

carotenoids in the extracts, and these will dominate the chromatogram in a fast analysis, too. Modern liquid chromategraphic instruments tolerate high flow rates and back pressures and enable the use of rapid resolution columns with smaller particle sizes that dramatically increase the chromatographic resolution. We choosed to use a single quadrupol (SQ) mass spectrometer for this analysis. The SQ MS has only unit mass resolution but this is for most cases enough to assign a carotenoid molecular formula since carotenoids have limited variation in chemical composition (i.e. molecules contain only C, H, and O). The advantage with quadrupol instruments is the option to run in Single Ion Monitoring (SIM) mode that filters away noise and increases sensitivity and selectivity. The SQ MS was set up with SIM for 20 carotenoid masses for 90% of the cycle time and 10% of the cycle time was run in scan mode. Data acquisition in scan mode was included in case a dominating peak in the UV/VIS-chromatogram could not be assigned to a mass in the SIM list. The SIM mass list was generated from analysis of the LC-TOF MS data on the sixteen isolates and a thorough analysis of all carotenoid entries in DNP (Supplementary data Table 2).

260 isolates were analyzed with the high throughput screening LC-MS method. They were chosen with a criterion of diversity and represented in total 8 different sampling points. The total number of pigments detected per isolate was as expected lower with the short run LC-MS method compared to the long run method (1-3 major pigments versus 3-5 major pigments – data not shown). Figure 3 shows the relative occurrence of carotenoids obtained by the LC-MS screen. Some masses appear at different retention times and clearly represent different carotenoids with the same molecular formula. Importantly, almost all pigments (>95%) could be assigned a carotenoid mass from the SIM-list implying that most of the pigments probably belong to the chemical group carotenoids. When the results from the screen were compared with the sampling data, there was a clear connection between salinity and number of different carotenoids per strain, with higher number of carotenoids and more intense coloration from isolates at medium salinity sampling sites. Regarding the surface microlayer; the high and low thickness range (0-10 µm and >31 µm) had isolates with relative more carotenoids than medium thickness layer.

Structural elucidation of major carotenoids with UVA-Blue light absorbing properties

For this study, we were particularly interested in carotenoids with yellow color and an UV/VIS absorbance profile covering the UVA-Blue range of the light specter. We defined λ_{max} below 450 nm as screening criterium, and all 260 UV/VIS chromatograms were thoroughly inspected. Six of the 260 isolates contained carotenoids with an UV/VIS absorbance profile significantly shifted downwards to the UVA-Blue range. Figure 4 shows the UV/VIS spectra of two major carotenoids in two of the six selected isolates. The comparison with astaxanthin visualizes the downward shift in the UV/VIS absorbance profile of the two selected carotenoids, and it can clearly be seen that these two carotenoids absorb in the UVA-Blue range while astaxanthin do not. The six strains were sent to NCIMB Ltd in Scotland for identification and Table 2 shows the outcome from the 16S rRNA analysis. The six isolates were further grown in 1 liter fermentors to produce enough biomass for purification of several UVA-Blue absorbing carotenoids by preparative HPLC. Pigment production varied

Table 2. Identification of the six strains producing UVA-Blue absorbing carotenoids by use of 16S rRNA analysis

Isolate identified as	Confidence (%)
Micrococcus luteus	99.6
Cyclobacterium sp.	99.4
Leeuwenhoekiella sp.	97.3
Xanthomonas sp.	99.6
Sphingomonas baekryungensis	99.8
Erythrobacter spp.	98.6

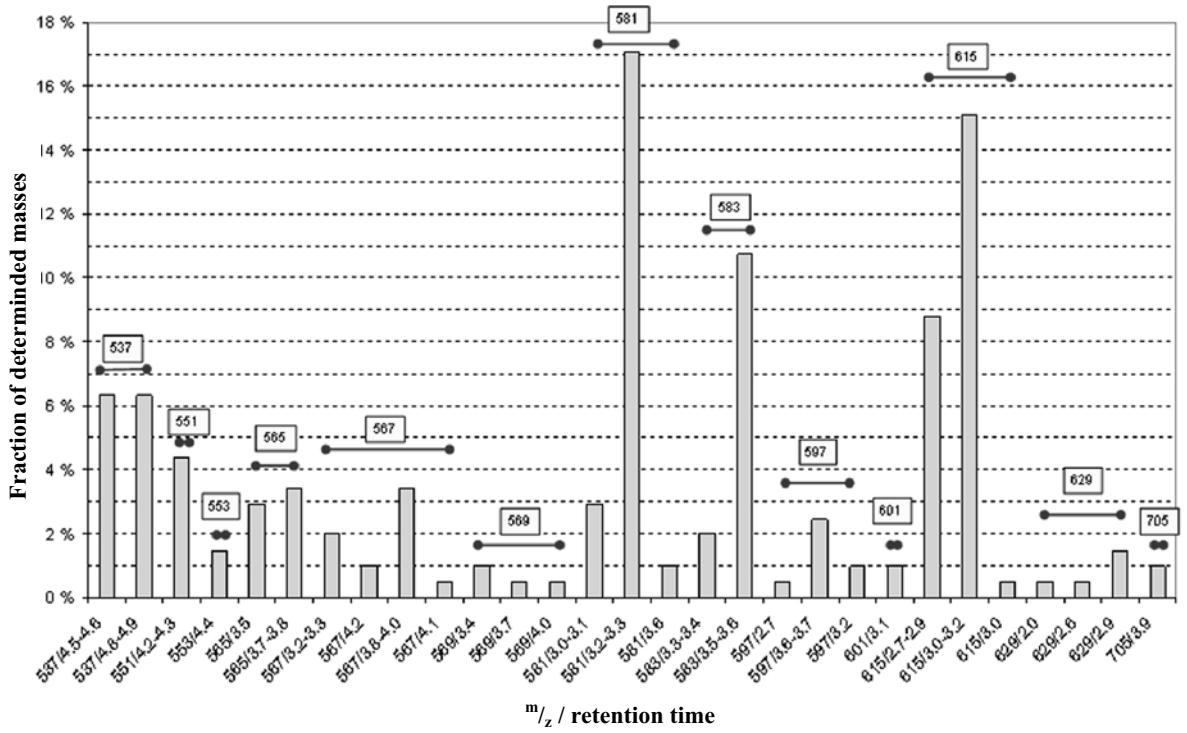

Fig. 3. Distribution of carotenoids identified by m/z and retention time during the high throughput LS-MS analysis of 260 different bacterial extracts. This represents all identified carotenoid masses from the SIM data acquisition and each column represents one carotenoid.

among the different isolates and the four isolates producing highest amounts were selected for purification of major pigments and subsequent structural elucidation using NMR.

Table 3 shows the tentative identification of carotenoids in this screening. Identification is based on ^{1}H NMR chemical shifts, provided in Supplementary data Tables 3 and 4 in the supplemental information, and their comparison with values reported elsewhere. The chemical shift values were for the most part obtained by identifying familiar patterns in the 2D ^{1}H,^{1}H COSY spectra, whereas the 1D ^{1}H spectra were mainly used as supporting evidence in the sugar region and for assignment of methyl groups. The assignments are incomplete for most of the carotenoids, due to the relatively small amounts of material available and lipid impurities. However, together with the UV/VIS and LC-MS data presented in Supplementary data Table 5, the combined evidence are in agreement with the proposed structures. For the diglycoside of nostoxanthin, there are some doubts as to how the two sugar moieties are connected to the carotenoid. Only one set of sugar signals is visible in the NMR spectrum, but integrals in the ^{1}H NMR spectrum suggest a 2:1 ratio between sugar and carotenoid. However, the carotenoid is unsymmetrical. This anomaly has not been resolved. Based on $^{3}J_{H,H}$ coupling constants, Supplementary data Table 3, the sugar is α-D-glucose.

For sarcinaxanthin diglycoside, the sugar moieties are also tentatively identified as α-D-glucose. Due to overlapping peaks from impurities and solvents in the ^{1}H NMR, no useful coupling patterns could be identified. The assignment is thus solely based on trends in the chemical shifts. However two mono- and diglucosides of sarcinaxanthin isolated from *Micrococcus luteus* are reported (Norgård *et al.*, 1970; Hertzberg and Liaaen-Jensen, 1977) and the reported values support our identification.

NMR data for the mixtures of zeaxanthin *cis*-isomers and nostoxanthin glycosides are not provided. However, structural elements related to the corresponding carotenoid end groups were present in ^{1}H,^{1}H COSY spectra of both mixtures, respectively. This combined with the available UV/VIS and LC-MS data provide the basis for these identifications.

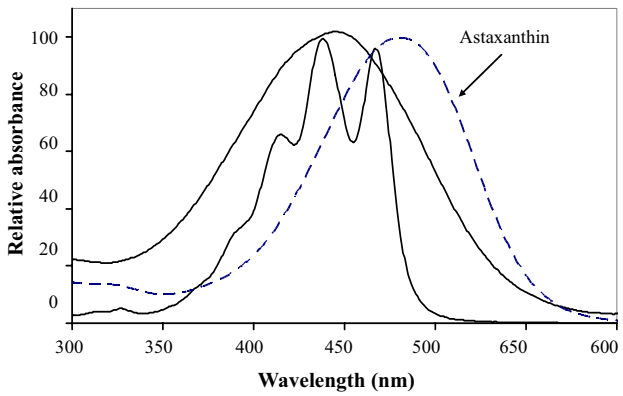

Fig. 4. UV/VIS-spectra of two of the identified carotenoids compared with astaxanthin standard (dashed line) to visualize the down-shift in λ_{max}.

Conclusion

We have established a collection of 1,500 pigmented marine

Table 3. Results from identification of carotenoids by NMR combined with MS and UV/VIS data

Isolate	# Pigment/isolate	Carotenoids identified		
Leeuwenhoekiella sp.	3	Zeaxanthin	9-*cis*-Zeaxanthin	13-*cis*-zeaxanthin
Spingomonas baekryungensis	5	4-ketonostoxanthin	Nostoxanthin	
Erythrobacter sp.	2	Nostoxanthin diglycoside[a]	Nostoxanthin[b]	
Micrococcus luteus	3	Sarcinaxanthin diglycoside	Sarcinaxanthin	

[a] A tentative identification

[b] Co-elution of at least three carotenoids, all nostoxanthin skeleton with sugar

bacteria and estimates suggest that there is approximately 1,000 unique isolates in the collection. Through initial characterization of 16 isolates with diode array detection and accurate mass determination, followed with a high throughput LC-MS screen of 260 isolates, we found a great diversity in carotenoids in general and also the number of different carotenoids per isolate. Through this work we have shown how valuable the collection is and how easy it is to access it and screen for carotenoids with particular interesting properties. Six promising isolates producing carotenoids with a low UV/VIS absorbance profile were selected for a detailed analysis including purification and structural elucidation of the most abundant carotenoids.

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