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Biochemical and Molecular Characterization
of Carotenogenic Flavobacterial Isolates from Marine Waters

RAMA SOWMYA and NAKKARIKE M. SACHINDRA*

Department of Meat and Marine Sciences, CSIR-Central Food Technological Research Institute
Mysore, India

Abstract

Carotenoids are known to possess immense nutraceutical properties and microorganisms are continuously being explored as natural source for production of carotenoids. In this study, pigmented bacteria belonging to *Flavobacteriaceae* family were isolated using kanamycin-containing marine agar and identified using the molecular techniques and their phenotypic characteristics were studied along with their potential to produce carotenoids. Analysis of random amplification of polymorphic DNA (RAPD) banding patterns and the fragment size of the bands indicated that the 10 isolates fall under two major groups. Based on 16S rRNA gene sequence analysis the isolates were identified as *Vitellibacter* sp. (3 isolates), *Formosa* sp. (2 isolates) and *Arenibacter* sp. (5 isolates). Phenotypically, the isolates showed slight variation from the reported species of these three genera of *Flavobacteriaceae*. Only the isolates belonging to *Vitellibacter* and *Formosa* produced flexirubin, a typical yellow orange pigment produced by most of the organisms of the family *Flavobacteriaceae*. *Vitellibacter* sp. and *Formosa* sp. were found to produce higher amount of carotenoids compared to *Arenibacter* sp. and zeaxanthin was found to be the major carotenoid produced by these two species. The study indicated that *Vitellibacter* sp. and *Formosa* sp. can be exploited for production of carotenoids, particularly zeaxanthin.

Introduction

Carotenoids are very widely distributed in nature, where they play an important role in protecting cells and organisms against the harmful effects of radicals. The primary mechanism of action is the ability of carotenoids to quench excited sensitizer molecules and quench singlet oxygen. Carotenoids can also serve as antioxidants under conditions other than photosensitization (Krinsky, 1989). Carotenoids from marine resources are known to possess various health beneficial activities (Sowmya and Sachindra, 2011).

Bacteria belonging to the phylum Cytophaga-Flavobacterium-Bacteroides (CFB) are common inhabitants of marine environments. Several *Flavobacterium* species are pathogenic to fish and invertebrates. *Flavobacterium columnare* causes columnaris disease (Decostere, 2002), and *Flavobacterium psychrophilum* causes infections in salmonid fish (Nematollahi *et al.*, 2003). Flavobacteria are characterized by their pigmentation properties, which is due to production of carotenoids. Zeaxanthin is the predominant carotenoid synthesized biologically by few species of the genus Flavobacterium (Johnson and Schroeder, 1995). Among the carotenoids present in the body, only lutein, zeaxanthin and meso-zeaxanthin, are found in the macula lutea of the eye. Lutein and zeaxanthin provide significant protection against the potential damage caused by light as reported in many studies (Bone *et al.*, 2007; Roberts *et al.*, 2009). It is also suggested that zeaxanthin is protective against age-related increase in lens density and cataract formation (Sajilata *et al.*, 2008). Liver carcinogenesis in C3H/He male mice was suppressed when fed with zeaxanthin mixed as an emulsion (Nishino *et al.*, 1999). Zeaxanthin plays an important role in the inhibition of macrophage-mediated LDL oxidation suggesting that it might help in slowing atherosclerosis progression (Carpenter *et al.*, 1997).

Much emphasis has been laid on the pathogenic Flavobacteria, but very little importance is given to the beneficial aspects of other bacteria belonging to *Flavobacteriaceae*. It has been reported that Flavobacteria produces not only zeaxanthin but also others carotenoids. Rählert *et al.* (2009) identified three carotenogenic

* Corresponding author: N.M. Sachindra, Department of Meat and Marine Sciences, CSIR-Central Food Technological Research Institute, Mysore, India; **e-mail: nmsachindra@cftri.res.in**

genes from *Flavobacterium* P99-3 that produces myxol, a monocyclic carotenoid and reported that *crt*A gene encodes for a novel carotenoid 2-hydroxylase involved in myxol biosynthesis which is highly homologous to *crt*A from purple bacteria encoding an acyclic carotenoid 2-ketolase. Another marine myxol-producing bacteria *Robiginitalea myxolifaciens* strain YM6-073 belonging to the *Flavobacteriaceae* have been isolated (Manh *et al.*, 2008). Saproxanthin and myxol isolated from *Flavobacteriaceae* are monocyclic carotenoids rarely found in nature and have antioxidative activities against lipid peroxidation in the rat brain homogenate model and neuro-protective effect from L-glutamate toxicity (Shindo *et al.*, 2007).

Eventhough, many new genera of CFB are described in the literature, their biochemical and molecular characterization is lacking, particularly the aspect of carotenoid production. In this study, the emphasis was given to the characterization of bacteria of the *Flavobacteriaceae* family from the Indian marine environment and carotenoid produced by them.

Experimental

Materials and Methods

Media and chemicals. Zobell marine broth, Zobell marine agar, Hi-carbokit and other media components were procured from Hi-Media Pvt. Limited. Mumbai, India. PCR components, lysozyme and primers were from Sigma-Aldrich, India. Solvents and analytical grade chemicals were from SRL, India. *Flavobacterium* sp. (MTCC 4664) was obtained from IMTECH, Chandigarh, India.

Selective isolation of bacteria of *Flavobacteriaceae* family. Samples of marine sediment, water from different depths, seaweeds and sponges were collected from different regions of East and west coast of India (Tuticorin, Mandappam, Rameshwaram and Mangalore). A selective marine agar medium containing kanamycin was used for isolation and enumeration of yellow-pigmented colonies by modified method of Flint (1985). Zobell marine broth containing kanamycin at 50 micrograms/ml concentration was used for isolation of bacteria from marine water and sediments samples. After incubation for 24 hrs, a loopful of sample was streaked onto marine agar plates containing kanamycin (50 µg/ml). Colonies showing the yellow-orange pigmentation were isolated and purified by repeated streaking.

Biochemical characterization. Biochemical tests performed include gram staining, motility test, oxidase activity, catalase production, deamination of L-tryptophan, decarboxylation of indole, MR-VP, oxidation and fermentation of sugars. Utilization of glucose, mannitol, inositol, sucrose and other 31 sugars was assessed by using Hi-carbo kit. Hydrolysis of esculin, gelatin and starch were also performed using standard techniques. Growth in marine broth at different temperatures of 10°C, 25°C, 37°C and 50°C, at different pH of 4,7 and 10 were performed to characterize the growth of organisms. Flexirubin production by the isolates was tested by the method of Fautz and Reichenbach (1980). Haemolytic activity of the cultures was tested using blood agar. Antibiotic sensitivity of the isolates was tested by the method of Bauer *et al.* (1966).

Scanning Electron Microscopic (SEM) analysis. Morphological analysis of the culture isolates were performed according to the method of McDougall *et al.* (1994). Briefly, selected isolates grown in marine broth were centrifuged, washed thrice with phosphate buffer saline (pH 7.0) to remove salts, fixed with glutaraldehyde (2%) and subjected to gradual alcoholic dehydration. The processed samples were then analyzed on SEM (Leo-435 VP, Leo Electron Microscope, Zeiss Ltd., Cambridge, UK).

Random Amplification of Polymorphic DNA (RAPD). Genomic DNA was extracted from the bacterial isolates using the standard DNA isolation protocol (Sambrook and Russell, 2001). Random amplification of polymorphic DNA (RAPD) was performed using M13 primer (5'-GAGGGTGGCGGTTCT-3') for microbial typing according to Schillinger *et al.* (2003) and the product was run on 1.8% agarose gel. RAPD banding pattern was further analyzed using Gene-Sys® software (SYNGENE, UK) and the similarity of the band profiles and the grouping of the RAPD-PCR patterns were calculated based on the Pearson's coefficient and agglomerative clustering with unweighted pairs group matching algorithm (UPGMA), and the dendrogram was constructed using GeneSys® software.

16S rRNA gene sequencing and phylogenic analysis. 16S rRNA gene was amplified using the universal bacterial forward and reverse primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (51-GGTTACCTTGTTACGACTT-31) respectively. Each PCR mixture of 25 µl contained template DNA (20 ng/µl), 0.2 µM of each primer, 0.25 mM of each deoxynucleoside triphosphate (dNTP), and 2.5 U of *Taq*DNA polymerase in a final concentration of 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$. PCR was performed under the following cycle conditions: an initial denaturation step at 94°C for 5 min and 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 2 min, and extension at 72°C for 2 mins. A final extension step at 72°C for 10 min was performed. The PCR product of 1.4 kb was purified and sequenced at Amnion Biosciences, Bangalore, India. The 16S rRNA gene sequences obtained was subjected to nBLAST

for similarity identification. Multiple alignment and sequence similarity with that of available sequences of reference strains from GenBank database were analyzed using CLUSTAL X. The phylogenetic tree was constructed using the MEGA 4 program (Biodesign Institute, Tempe, AZ, USA) by the method of neighbor-joining (Kumar *et al.*, 2008). The sequences have been deposited at NCBI GenBank.

Whole-cell protein analysis. Whole-cell protein extracts were prepared by suspending the culture pellet in phosphate buffer saline (pH 7.0) and sonicating at 150 W power until the suspension is clear (Chart, 1994). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the whole-cell protein extracts was performed with a 12% gel by the procedure of Laemmli (1970) and run at a constant current of 16 mA, stained overnight with coomassie brilliant blue R-250 and following destaining the electrophoretic patterns were scanned with a gel documentation system (G-Box, SYNGENE, UK) and cluster analysis of electrophoretic patterns was done by the software GeneSys®. A dendrogram representing the relationship between all strains tested was derived from the TREE option. The whole-cell protein patterns of the 10 isolates and standard culture were compared.

Extraction and characterization of carotenoids. The bacterial isolates were inoculated into 50 ml marine broth and grown for 5 days at 37°C under shaking condition (200 rpm) and the cells were harvested by centrifuging at 8000 rpm for 15 min. Cells were suspended in sterile saline and lysed by adding the lysozyme (20 mg/ml). Carotenoids were extracted by homogenizing the lysed cell suspension with acetone, filtered, and cells re-extracted with fresh acetone. The acetone extract was pooled and subjected to phase separation with equal quantity of hexane. The hexane extract was dried with sodium sulphate to remove traces of water if any, filtered and then evaporated under vacuum at 40°C using a rotary flash evaporator and flushed with

nitrogen to obtain the total carotenoid extract. The yield of recovered carotenoid was determined.

Thin layer chromatography (TLC) of carotenoid extract from selected isolates was performed using Silica Gel 60 plates (MERCK). The samples along with standard carotenoids and plates were developed using the mobile phase of acetone: hexane (75:25). Then, HPLC of the extracted carotenoids was done by C18 TSK gel ODS-80TS column (4.6 mm ID × 25 cm) with gradient run of 35 min with solvent A as methanol and water (95:5) and solvent B as methanol and tetrahydrofuran (7:3) with a flow rate of 1.25 ml/min at 470 nm using a diode array detector.

Statistical analysis. Mean values of yield of crude carotenoid extract and zeaxanthin content in the carotenoid extract were subjected to ANOVA to determine the effects, and mean differences observed were subjected to Duncan's multiple range test using the software STATISTICA (Statsoft, 1999).

Results and Discussion

Isolation and characterization of carotenoid-producing bacteria. Pigmented colonies were selected from the marine agar plates containing the kanamycin and were purified. In all 10 isolates from different location and source were selected for further study based on pigmentation, source and location (Table I). The color of the selected isolates was yellow-orange to dark orange, and all the isolates had a slimy surface. The 10 selected cultures along with the standard culture *Flavobacteria* sp. MTCC 4664 had distinct RAPD banding patterns, and the fragment size of the bands ranged from 250 to 2370 bp. According to the dendrogram (Fig. 1) and cluster analysis, the isolates were grouped into two major groups, wherein isolates KW1, KW2, SUW, 4W and 7W were in one group and the other 5 isolates along with MTCC 4664 were in the other group.

Table I
Details of isolates

Isolate	Source	Location	Colony characteristics
BW	Sea water (beach side)	13.87°N, 74.63°E	Dark orange, slimy
C12	Sea water (12 meter depth)	12.57°N, 74.47°E	Dark orange, slimy
NMW	Mangrove water	12.80°N, 74.85°E	Dark orange, slimy
MBW	Sea water (beach side)	13.35°N, 74.70°E	Orange, slimy
KMW	Mangrove water	13.21°N, 75.02°E	Orange, slimy
4W	Sea water (beach side)	08.53°N, 78.36°E	Yellow orange, slimy
7W	Sea water (beach side)	08.48°N, 78.12°E	Yellow orange, slimy
KW1	Sea water (beach side)	13.49°N, 74.70°E	Yellow orange, slimy
KW2	Sea water (beach side)	13.49°N, 74.70°E	Yellow orange, slimy
SUW	Sea water (beach side)	12.98°N, 74.78°E	Yellow orange, slimy

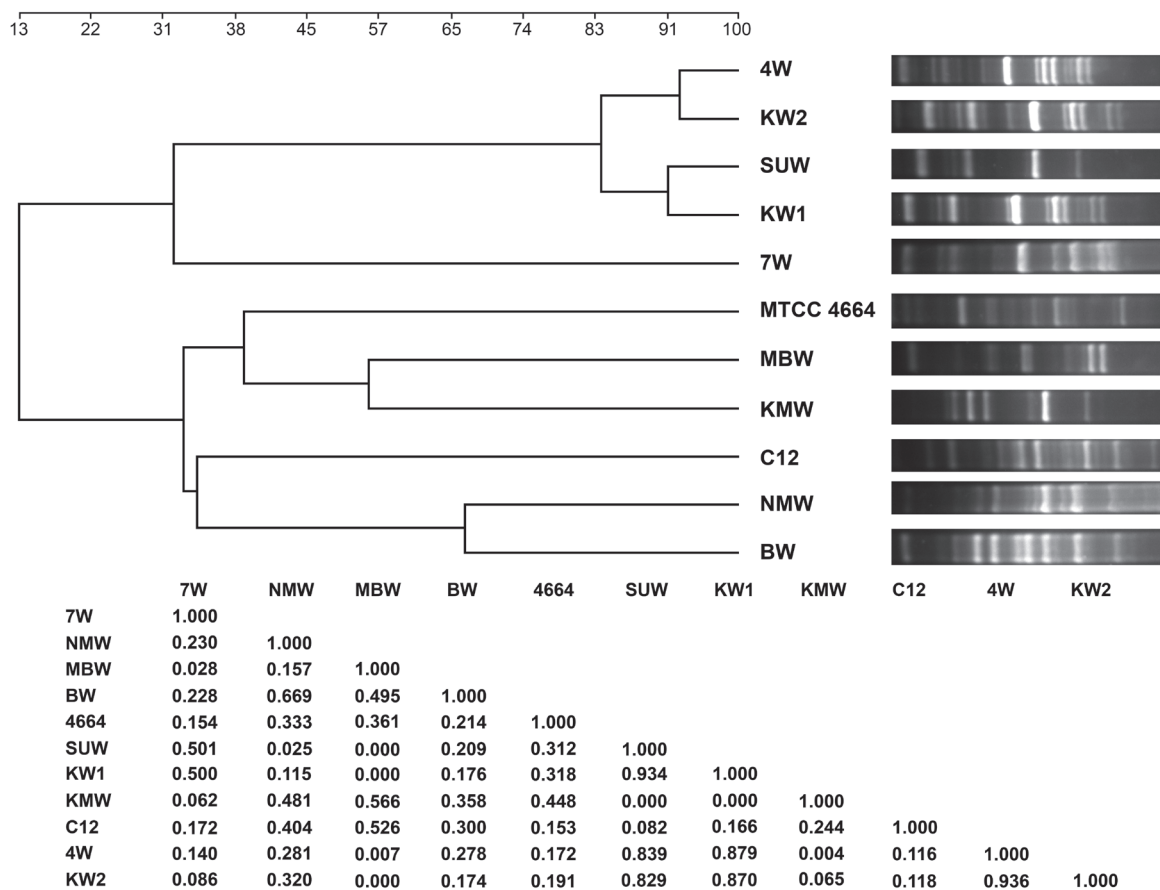|	7W	NMW	MBW	BW	4664	SUW	KW1	KMW	C12	4W	KW2
7W	1.000										
NMW	0.230	1.000									
MBW	0.028	0.157	1.000								
BW	0.228	0.669	0.495	1.000							
4664	0.154	0.333	0.361	0.214	1.000						
SUW	0.501	0.025	0.000	0.209	0.312	1.000					
KW1	0.500	0.115	0.000	0.176	0.318	0.934	1.000				
KMW	0.062	0.481	0.566	0.358	0.448	0.000	0.000	1.000			
C12	0.172	0.404	0.526	0.300	0.153	0.082	0.166	0.244	1.000		
4W	0.140	0.281	0.007	0.278	0.172	0.839	0.879	0.004	0.116	1.000	
KW2	0.086	0.320	0.000	0.174	0.191	0.829	0.870	0.065	0.118	0.936	1.000

Fig. 1. Dendrogram drawn using RAPD profile of bacterial isolates.
UPGMA bootstrapping values of this clade were 95–99. Culture isolates were grouped by considering the significances
in bootstrap values, interior branch lengths, and diversification rate.

The second group consisted of three subgroups having isolates NMW and BW in one subgroup and C12, KMW and MBW in another subgroup, with MTCC 4664 being a separate subgroup.

The whole-cell protein patterns of the 10 isolates with the standard culture had a major protein band patterns ranging from 18 kDa to 116 kDa. Heterogeneity in SDS-PAGE protein patterns among different isolates was clearly observed. A dendrogram was created based on the electrophoretic pattern and is depicted in Fig. 2. Two major groups were formed based on SDS-PAGE patterns of whole cell protein, where the isolates MBW, KMW along with MTCC 4664 isolates formed one group and the rest being in the other group. Among the 8 isolates in the second group, a high similarity of 0.891 was observed between the isolates SUW and KW1.

The selected isolates were identified by 16S rRNA gene amplification. The 16S rRNA gene products were sequenced, and the sequences were used for database query. The nucleotide sequence data has been deposited at the National Center for Biotechnology Information (NCBI). The 16S rRNA gene sequences were subjected for BLAST using the megablast tool of GenBank (http://www.ncbi.nlm.nih.gov/), which revealed that the iso-lates belonged to three genera of *Flavobacteriaceae*, namely, *Formosa*, *Vitellibacter* and *Arenibacter*. The isolates under genus *Arenibacter* along with their GenBank accession number are *Arenibacter* sp. SUW (KJ658267), *Arenibacter* sp. KW1 (KJ658268), *Arenibacter* sp. 4W (KJ658269), *Arenibacter* sp. KW2 (KJ658270) and *Arenibacter* sp. 7W (KJ658274). The three *Vitellibacter* strains are *Vitellibacter* sp. C12 (KJ658271), *Vitellibacter* sp. NMW (KJ658275), *Vitellibacter* sp. BW (KC888001) and the two *Formosa* strains were *Formosa* sp. KMW (KC888002) and *Formosa* sp. MBW (KC888003).

Representatives of maximum homologous (98–99%) sequences of each isolate and other members of the Flavobacteria family like *Aequorivita, Olleya, Yeosuana, Lacinutrix* and *Flexibacter* were obtained from NCBI GenBank and were used for the construction of a phylogenetic tree which is rooted where a common ancestor is defined. All the 5 *Arenibacter* isolates were found to be grouped along with *Arenibacter latericius, Arenibacter certessi* and *Flexibacter aggregans*. All three *Vitellibacter* isolates were in a group with *Vitellibacter vladivostokensis* and *Aequorivita* sp., while *Formosa* isolates were in the group comprising of *Oleya* sp., *Yeosuna* sp., *Gaelibulibacter* sp., *Gelidibacter* sp. and *Bizionia* sp. (Fig. 3).

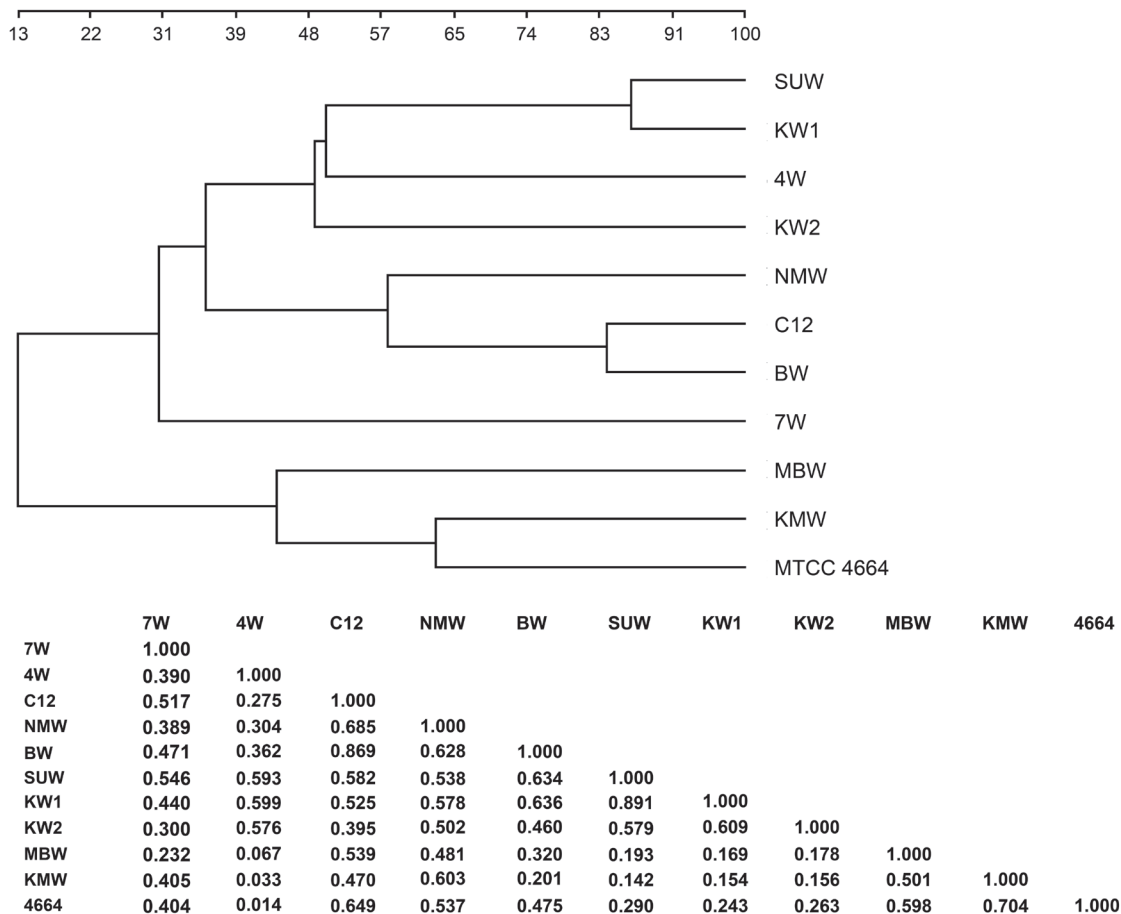|	7W	4W	C12	NMW	BW	SUW	KW1	KW2	MBW	KMW	4664
7W	1.000										
4W	0.390	1.000									
C12	0.517	0.275	1.000								
NMW	0.389	0.304	0.685	1.000							
BW	0.471	0.362	0.869	0.628	1.000						
SUW	0.546	0.593	0.582	0.538	0.634	1.000					
KW1	0.440	0.599	0.525	0.578	0.636	0.891	1.000				
KW2	0.300	0.576	0.395	0.502	0.460	0.579	0.609	1.000			
MBW	0.232	0.067	0.539	0.481	0.320	0.193	0.169	0.178	1.000		
KMW	0.405	0.033	0.470	0.603	0.201	0.142	0.154	0.156	0.501	1.000	
4664	0.404	0.014	0.649	0.537	0.475	0.290	0.243	0.263	0.598	0.704	1.000

Fig. 2. Dendrogram derived from the UPGMA clustering of correlation coefficients based on the SDS-PAGE of whole-cell protein patterns of 10 Flavobacterial isolates along with standard culture (MTCC 4664).

The phenotypic characteristics of all the isolates are given in Table II. All the isolates were gram-negative rods with yellow-orange colored slimy colonies. The selected isolates were non-motile, asporogenic, indole-, citrate-, malonate- and MRVP-negative and catalase-positive. Except three isolates of *Vitellibacter* all the other isolates were positive for oxidase. All strains were negative for fermentation and hence, they are strictly aerobic. The isolates belonging to *Vitellibacter* and *Formosa* were not able to produce acids from any sugars while those belonging to *Arenibacter* genus were able to produce acid from many of the sugars. *Vitellibacter* sp. BW and two *Formosa* isolates were able to hydrolyse gelatin. The optimum growth of these marine organisms was at pH 7.0. However, some of the isolates could grow at pH 4.0. *Arenibacter* sp. 4W showed weak growth at pH 10.0. All the isolates were able to grow at 37°C; however the isolates belonging to *Vitellibacter* showed better growth and pigmentation at 25°C. All the isolates were found to be nonpathogenic as tested by haemolytic activity. With respect to antibiotic sensitivity of isolates, all the isolates were sensitive to most of the antibiotics tested. However, 5 of the isolates were resistant to tobramycin (data not shown) indicating that

tobramycin could also be used in the media for selective isolation and to prevent contamination from other bacteria when used for carotenoid production.

SEM images of the bacteria give a clear image of the structural features of the bacteria. The SEM images of the different *Flavobacteriaceae* isolates are shown in Fig. 4. Isolates belonging to *Vitellibacter* were thin elongated rod-shaped bacteria without flagella or fimbriae. The isolates belonging to *Formosa* were short rods, while those belonging to *Arenibacter* were moderately long rods.

Production of yellow or orange pigments on agar plates has been used as one of the criteria for isolation of the members of the family *Flavobacteriaceae*. However, the overgrowth of non-pigmented bacteria, particularly from soil samples necessitates use of selective media for isolation of flavobacteria. As flavobacteria are known to be resistant to kanamycin, Flint (1985) suggested the use of kanamycin-containing medium for selective isolation of flavobacteria. In the present study when kanamycin-containing marine agar was used for isolation of bacteria from marine sources, the dominance of pigment producers in the media was observed. The pigment producing isolates were purified for further

Table II
Phenotypic characteristics of the isolates.

	Vitellibacter sp. C12	*Vitellibacter* sp. BW	*Vitellibacter* sp. NMW	*Formosa* sp. KMW	*Formosa* sp. MBW	*Arenibacter* sp. 4W	*Arenibacter* sp. 7W	*Arenibacter* sp. KW1	*Arenibacter* sp. KW2	*Arenibacter* sp. SUW
Gram reaction	–	–	–	–	–	–	–	–	–	–
Morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Oxidase	–	–	–	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
MR/VP	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
Indole	–	–	–	–	–	–	–	–	–	–
O/F	–/–	–/–	–/–	–/–	–/–	+/–	+/–	+/–	+/–	+/–
Flexirubin production	+	+	+	+	+	–	–	–	–	–
Acid from										
Lactose	–	–	–	–	–	+	+	+	+	+
Xylose,	–	–	–	–	–	+	+	+	–	+
Maltose	–	–	–	+	+	+	+	+	+	+
Fructose	–	–	–	–	–	+	+	+	+	+
Dextrose	–	–	–	+	+	+	+	+	+	+
Galactose	–	–	–	–	–	+	+	+	+	+
Raffinose	–	–	–	–	–	+	+	+	+	+
Trehalose	–	–	–	+	+	+	+	+	+	+
Melibiose	–	–	–	–	–	+	+	+	+	+
Sucrose	–	–	–	w	w	+	+	+	+	+
L-Arabinose	–	–	–	–	–	–	–	–	–	–
Mannose	–	–	–	+	+	–	–	–	–	–
Inulin	w	w	w	w	w	+	+	+	+	+
Sodium gluconate	–	–	–	–	–	+	+	–	+	+
Glycerol	–	–	–	–	–	–	–	–	–	–
Salicin	–	–	–	+	+	–	–	–	+	–
Dulcitol	–	–	–	–	–	+	+	+	+	+
Inositol	–	–	–	w	w	–	–	–	+	–
Sorbitol	–	–	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	–	–	–	–
Adonitol	–	–	–	–	–	–	–	–	–	–
Arabitol	–	–	–	–	–	–	–	+	–	+
Erythritol	–	–	–	–	–	–	–	–	–	–
α-Methyl-D-glucoside	–	–	–	–	–	–	–	–	+	–
Rhamnose	–	–	–	–	–	+	+	+	+	+
Cellobiose	–	–	–	w	w	–	–	–	–	–
Melezitose	–	–	–	–	–	+	+	+	+	+
α-Methyl-D-Mannlside	–	–	–	–	–	+	+	+	–	+
Xylitol	–	–	–	–	–	+	+	+	+	+
D-arabinose	–	–	–	–	–	+	+	+	+	+
Sorbose	–	–	–	–	–	–	–	–	–	–
β-galactosidase	–	–	–	–	–	–	–	–	–	–

Table II. Continued.

	Vitellibacter sp. C12	Vitellibacter sp. BW	Vitellibacter sp. NMW	Formosa sp. KMW	Formosa sp. MBW	Arenibacter sp. 4W	Arenibacter sp. 7W	Arenibacter sp. KW1	Arenibacter sp. KW2	Arenibacter sp. SUW
Hydrolysis of										
Esculin	–	–	–	–	–	–	–	–	–	–
Gelatin	–	+	–	+	+	–	–	–	–	–
Starch	–	–	–	–	–	–	–	–	–	–
Citrate Utilization	–	–	–	–	–	–	–	–	–	–
Malonate Utilization	–	–	–	–	–	–	–	–	–	–
Growth at pH										
4.0	–	w	–	+	–	w	–	w	w	+
7.0	+	+	+	+	+	+	+	+	+	+
10.0	–	–	–	–	–	w	–	–	–	+
Growth at temperature										
25°C	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+
50°C	–	–	–	w	w	–	–	–	–	–
Haemolysis	–	–	–	–	–	–	–	–	–	–

+: Positive; –: Negative; W: Weak

characterization using phenotypic and molecular techniques like Random Amplified Polymorphic DNA (RAPD) finger printing 16S rRNA gene sequencing.

RAPD is a relatively rapid PCR-based genomic fingerprinting method and is a useful tool for genome analysis in bacterial identification where isolates can be compared and grouped. Polymorphism between strains is detected as the difference between the patterns of amplified DNA fragments. In the present study the analysis of RAPD banding patterns of the isolates by dendrogram and cluster analysis indicated that the 10 isolates were in two major groups. Five isolates, which were later identified as *Arenibacter* sp. formed one group and the other 5 isolates later identified as either *Vitellibacter* sp. or *Formosa* sp. formed another group. The results indicated that genetic homogeneity between *Vitellibacter* sp. and *Formosa* sp. and their diversity from *Arenibacter* sp. under the *Flavobacteriaceae* family.

Whole-cell protein pattern analysis by SDS-PAGE and dendrograms obtained from numerical analysis is one of the techniques used for typing of bacterial species and grouping (Walia *et al.*, 1988). In the present study analysis of whole cell protein from the different isolates using SDS-PAGE indicated that based on banding pattern the isolates can be clubbed into two major groups. However, grouping was not similar to grouping based on RAPD fingerprinting. Even the isolates belonging to same genera were in different groups, indicating that SDS-PAGE of whole cell protein is not a reliable tool for grouping of selected isolates.

The phenotypic characteristics of the bacterial isolates provide information about nutrient and environmental requirement for the growth of the organisms, and such information will be useful when the organisms used for exploiting their beneficial role. The phenotypic characteristics of the isolates indicated that they are able to use different sugars and optimum growth conditions were found to be pH 7.0 and a temperature range of 25–37°C.

Analysis of 16S rRNA gene sequences of the selected isolates indicated that they belong to three genera of *Flavobacteriaceae*, namely, *Formosa*, *Vitellibacter* and *Arenibacter*. Isolation and characterization of organisms belonging to these three genera from different sources has been reported. Two species of *Formosa* genus, *Formosa algae* KMM3553 (Ivanova *et al.*, 2004) and *Formosa agariphila* (Nedashkovaskaya *et al.*, 2006a) has been reported so far. *F. algae* isolated from degrading thallus of the brown alga *Fucus evanescens* was found to be light yellow pigmented and positive for gliding motility, catalase, urease and utilization of lactose and glycerol and oxidase negative (Ivanova *et al.*, 2004), while *F. agariphila* isolated from green alga *Acrosiphonia sonderi* and from sea water was positive for gliding motility, oxidase, catalase and for sugar utilization.
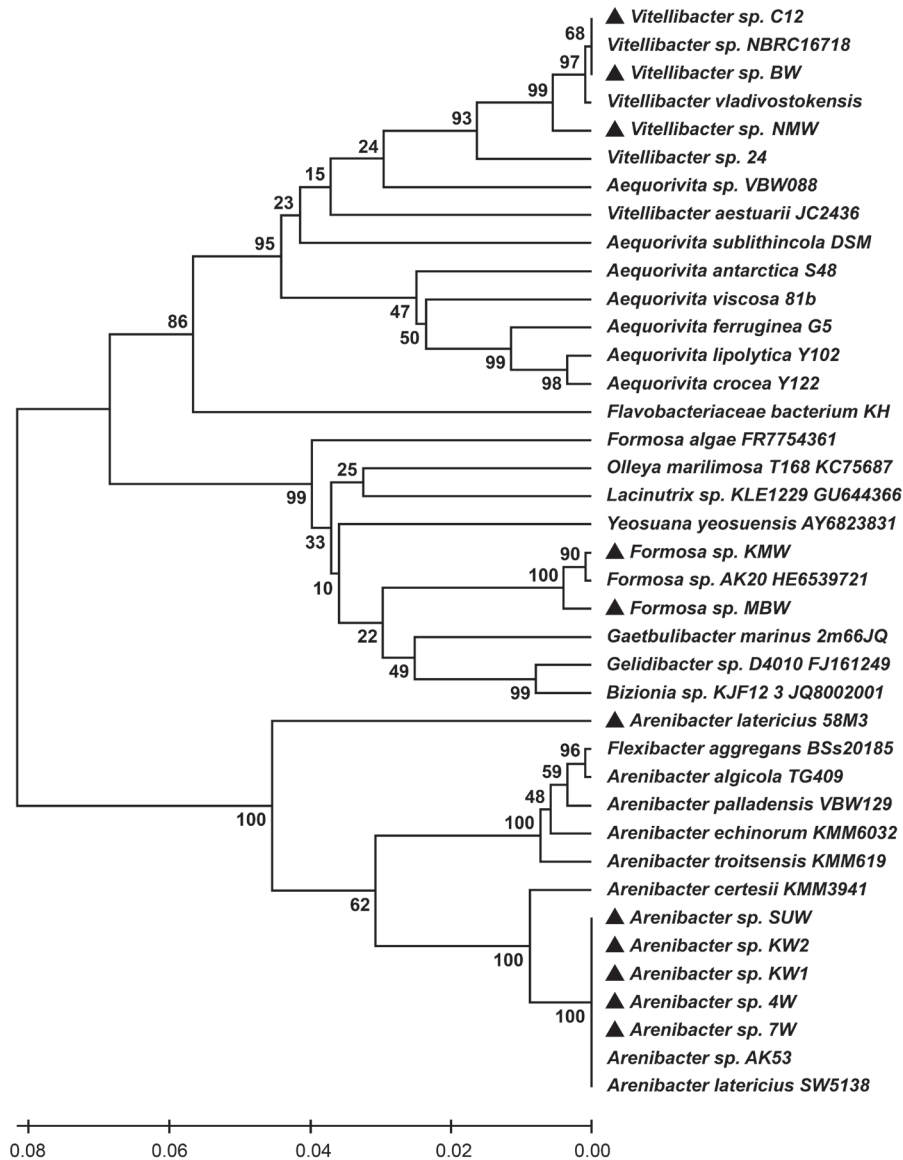
Fig. 3. Phylogenetic analysis of isolated cultures based on 16S rRNA gene sequence.

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Phylogenetic analyses was conducted by MEGA4 software. Representative sequences were obtained from GenBank and accession numbers given in parentheses. Flavobacterial cultures isolated are represented by

However, the two isolates of *Formosa* identified in the present study are phenotypically different indicating that they may be different species of *Formosa*.

The three species of *Vitellibacter* genus reported are *Vitellibacter vladivostokensis* KMM3516 (Nedashkovskaya *et al.*, 2003a), *Vitellibacter aestuarii* JC2436 (Kim *et al.*, 2010), and *Vitellibacter soesokkakensis* (Park *et al.*, 2014). *V. vladivostokenisis* isolated from holothurian *Apostichopus japonicus* collected from Troitsa Bay in the Sea of Japan were yellow-orange pigmented, non-motile, asporogenic, and strictly aerobic, gram-negative, oxidase- and catalase-positive bacteria. *V. aestuarii* isolated from tidal-flat sediment of Oi Island in Korea

was found to be closely affiliated to *V. vladivostokensis*, with 96% sequence similarities to the type strain. The difference between the two strains was found to be *V. vladistokensis* being catalase-negative and susceptible to benzylpencillin but not to ampicillin and lincomycin (Kim *et al.*, 2010). The *Vitellibacter* sp. isolated in the present study does not match exactly with the known *Vitellibacter* species reported and hence may be different species of *Vitellibacter*.

Presently six species of *Arenibacter* have been reported. They are *Arenibacter latericius* isolated from marine sediments (Ivanova *et al.*, 2001), *Arenibacter troitsensis* isolated from marine bottom sediments
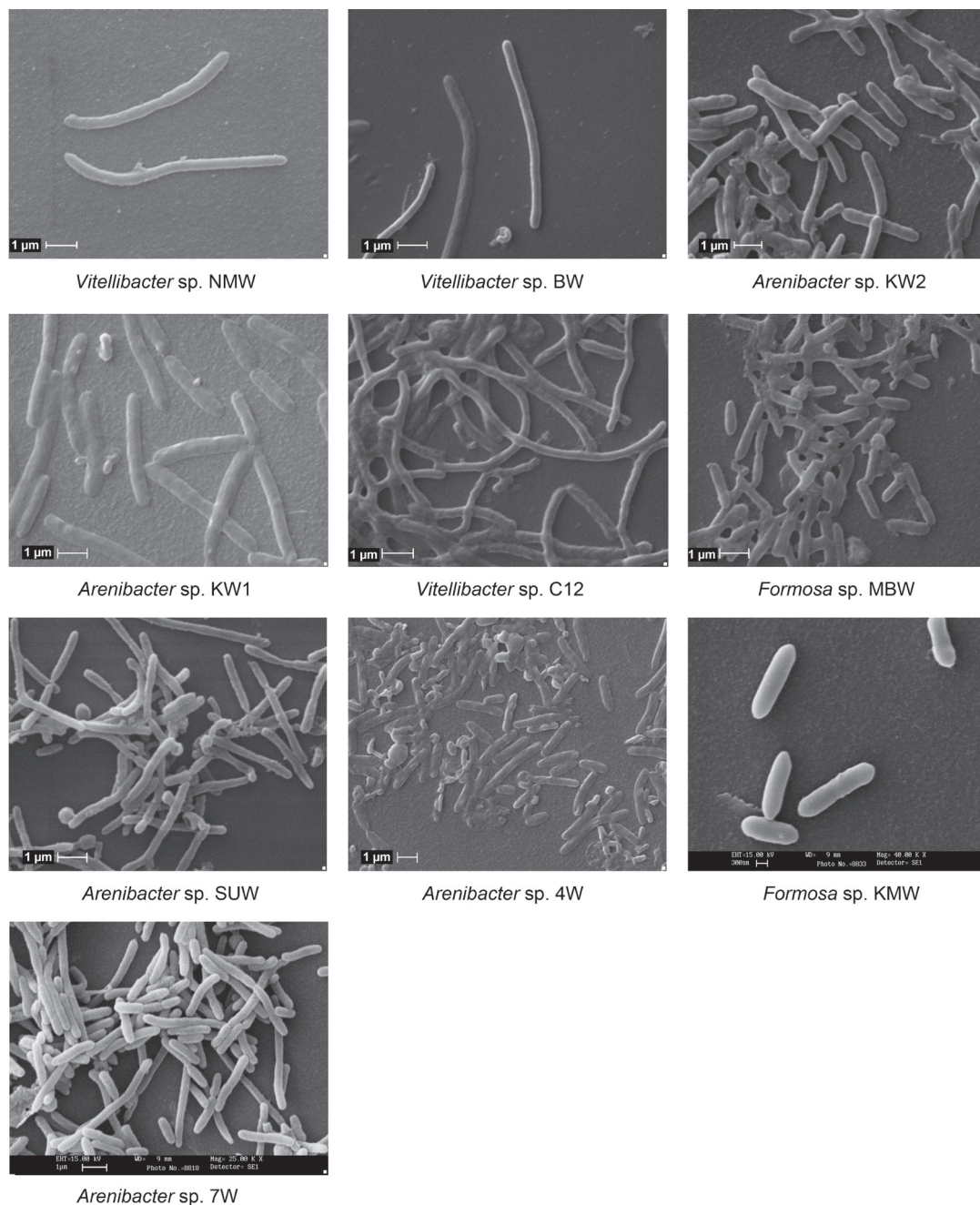
Fig. 4. SEM image of Flavobacterial isolates depicting their structural morphology.

(Nedashkovskaya *et al.*, 2003b), *Arenibacter certessi* isolated from green algae (Nedashkovskaya *et al.*, 2004), *Arenibacter palledensis* isolated from green algae (Nedashkovskaya *et al.*, 2006b), *Arenibacter echinorum* isolated from sea urchin (Nedashkovskaya *et al.*, 2007) and *Arenibacter nanhaiticus* from marine sediment (Sun *et al.*, 2010). Nedashkovskaya *et al.* (2007) compared the phenotypic characteristics of five species of *Arenibacter* and reported that they vary with respect to motility, production of acids from sugar, and gelatin hydrolysis. Three species (*A. echinoum*, *A. latericius* and *A. palledensis*) were able to produce acid from many of the sugars, and *A. echinorum* was able to utilize

malonate and citrate, but not the other isolates. In the present study also all the *Arenibacter* isolates were able to produce acids from many of the sugars, but many other phenotypic characteristics did not match with the known isolates.

Extraction and characterization of carotenoids. The total carotenoid yield from different Flavobacterial isolates varied (Fig. 5). Significantly ($p < 0.05$) higher carotenoid yield (115.7 ± 5.0 mg/g biomass) was observed in *Vitellibacter* sp. BW, followed by *Arenibacter* sp. 4W (99.3 ± 21.9 mg/g biomass). There was no specific dependency between carotenoid yield and the specific genus of the isolate.
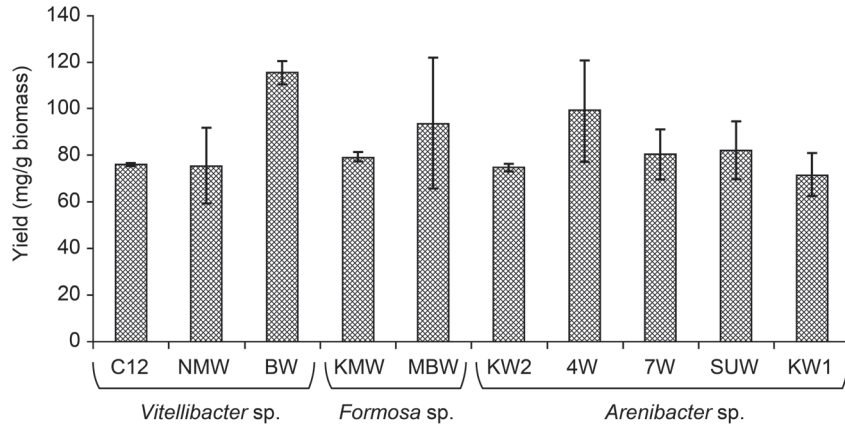
Fig. 5. Yield of crude carotenoid extract from different isolates.
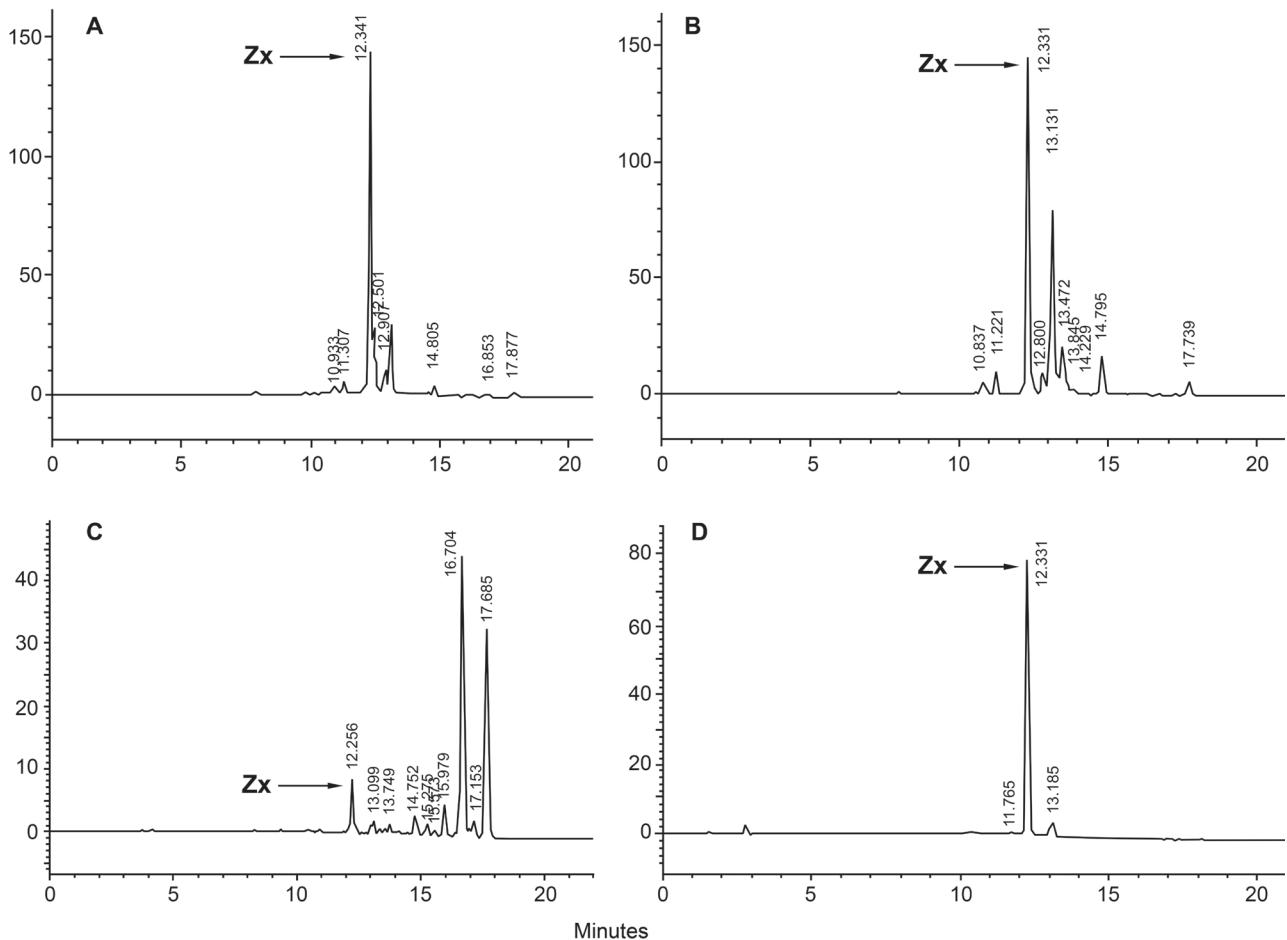
Fig. 6. HPLC profile of carotenoid extract of: (A) *Vitellibacter* sp. NMW; (B) *Formosa* sp. MBW; (C) *Arenibacter* sp. 4W; (D) Zeaxanthin (Zx) standard.

The preliminary test for the identification of carotenoids in the crude carotenoid extract by TLC showed the presence of β-carotene and zeaxanthin. HPLC analysis of crude carotenoid extract from all isolates confirmed the presence of zeaxanthin and β-carotene, however, the concentration of two major carotenoids differed with the isolates. The typical HPLC profiles of one isolate from the three genera *Vitellibacter* NMW,

Formosa MBW and *Arenibacter* 4W are given in figure 6A, 6B and 6C respectively and the HPLC profile of zeaxanthin standard in figure 6D. Significantly higher ($p < 0.05$) zeaxanthin concentration of $61.4 \pm 8.3\%$ was observed in the crude carotenoid extract from *Formosa* sp. KMW, followed by 59.6 ± 18.8 and $52.2 \pm 20.2\%$ in carotenoid extract from *Vitellibacter* sp. BW and C12 (Fig. 7). The zeaxanthin content in the carotenoid extract
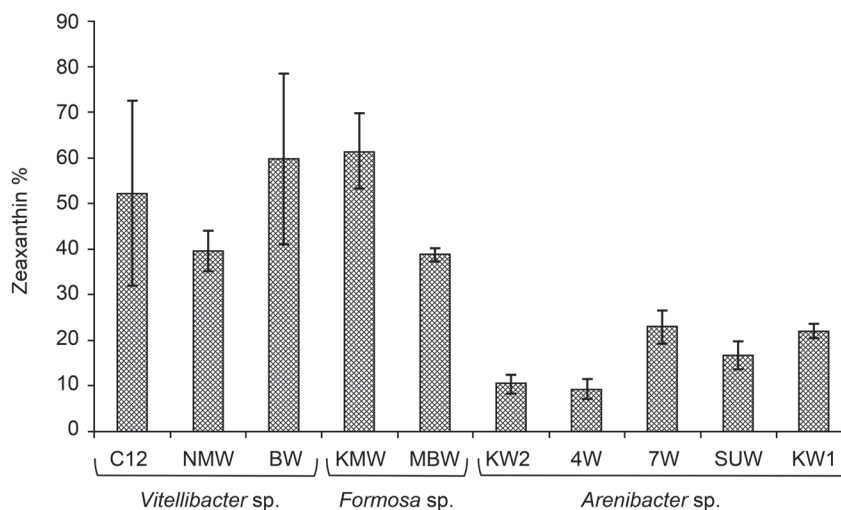
Fig. 7. Percentage of zeaxanthin in carotenoid extract from different Flavobacterial isolates.

from *Arenibacter* sp. was comparatively lower and was in the range of 9.2 to 23.0% of total carotenoid content.

Organisms belonging to *Flavobacteriaceae* are well known for the production of pigments. Flavobacterial isolates from soil and fresh water are known to produce flexirubin, a yellow-orange pigment, which is used as a taxonomic marker (Reichenbach *et al.*, 1980). In the present study, only the isolates belonging to *Vitellibacter* and *Formosa* were found to produce flexirubin pigment (Table II). However, yellow pigmentation of marine isolates has been mainly attributed to carotenoids, particularly zeaxanthin (Stafsnes and Bruheim, 2013). Studies on the carotenoid production by *Vitellibacter* sp., *Formosa* sp. and *Arenibacter* sp. is lacking. As carotenoids have been shown to provide various health benefits, the isolates in the present study were tested for production of carotenoids with an aim to select a suitable isolate for further investigation on carotenoid production.

Studies on carotenoid production by *Flavobacterium multivorum* indicated that the organism produces zeaxanthin as the major carotenoid (Alcantara and Sanchez, 1999; Bhosale *et al.*, 2004; Masetto *et al.*, 2001). However, under specific nutrient condition such as the presence of inorganic salts and urea in the growth media interferes with the zeaxanthin biosynthesis pathway and results in the accumulation of intermediates such as canthaxanthin and β-carotene (Bhosale and Bernstein, 2004). The present study indicated that the isolates *Vitellibacter* sp. BW and C12 and *Formosa* sp. KMW can be successfully exploited as zeaxanthin producers.

In conclusion, the study resulted in selective isolation and identification of 10 isolates of carotenoid producing *Flavobacteriaceae* belonging to three genera of *Arenibacter*, *Formosa* and *Vitellibacter*. Since, not much information is present on these organisms and on the carotenoid production aspects of *Vitellibacter* sp., *Formosa* sp. and *Arenibacter* sp. an attempt has been made

to study them. *Vitellibacter* sp. and *Formosa* sp. was found to produce higher amount of carotenoids compared to *Arenibacter* sp. Further, zeaxanthin was found to be the major carotenoid produced by *Vitellibacter* sp. and *Formosa* sp. These isolates have potential to be used for production of zeaxanthin in large scale that needs further studies on factors influencing zeaxanthin production by these isolates.

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