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Characterization of Canthaxanthin Isomers Isolated from a New Soil *Dietzia* sp. and Their Antioxidant Activities S

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Canthaxanthin (cx) is a potent antioxidant that is chemically synthesized at the industrial scale and has imperative applications in the cosmetic and feed industries. An orange pigmented mesophilic bacterium, designated as K44, was isolated from soil samples of Kargil, India. Biochemical tests, 16S rRNA gene sequencing, and FAME analysis of the bacterium indicated it to belong in the genus *Dietzia* and is distinct from human isolates. The strain showed 98% 16S rRNA gene sequence homology with *Dietzia maris* DSM 43102. High-performance liquid chromatography profile of the pigments isolated from K44 showed two major peaks absorbing at 465.3 and 475 nm. The liquid chromatography-mass spectrometry (LC-MS) analysis of both these peaks revealed their *m/z* to be 564. The molecular weights, LC-MS/MS fragmentation patterns, and λ_{max} of these fractions corresponded to all-*trans*-(475 nm) and 9-*cis*-(465.3 nm) cx isomers. The antioxidant activities of *cis*- and *trans*-cx isomers isolated from this bacterium were found to differ, where the *cis*-isomer showed higher free radical, superoxide radical, and reactive oxygen species scavenging activities than the all-*trans*-isomer, suggesting that 9-*cis*-cx is more effective as an antioxidant than the all-*trans*-cx.

Key words: *Dietzia* sp. K44, antioxidant, canthaxanthin, LC-MS/MS

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S Supplementary data for this paper are available on-line only at http://jmb.or.kr.

The genus *Dietzia* was first proposed on the basis of phylogenetic analysis to accommodate strains previously classified as *Rhodococcus maris* [32]. Subsequently, *Dietzia* strains have been found to inhabit a wide variety of environments including deepest sea mud [36], oral microbiota of domestic dogs [14], the pupae of an obligate parasitic fly, *Wohlfahrtia magnifica* [39], and petroleum contaminated soil [43]. All the strains assigned to the genus *Dietzia* have been reported to be pigmented, their color ranging from yellow to coral-red [12, 17, 25, 45, 46], but production of canthaxanthin (cx) was not reported in the *Dietzia* species except by our group in *Dietzia* sp. K44 [41] and by Khodaiyan *et al*. [18] in *Dietzia natronolimnaea* HS-1.

Cx is a diketocarotenoid (β,β-carotene-4,4-dione), occurring naturally in a wide variety of living organisms, where it plays important roles in animal displays of maturity and in the protection of plant and animal tissues against oxidizing free radicals [26]. The conjugated double-bond structure is responsible for its characteristic color as well as the antioxidant properties. It has been demonstrated to be a more potent antioxidant than β-carotene or zeaxanthin [38]. However, most of the research on cx has employed only the all-*trans* isomer, with the exception of the studies on brine shrimp *Artemia*, wherein *cis*-cx was isolated from the ovaries of female *Artemia*, attributing its possible role in reproduction and/or embryonic development [30].

Cx is used extensively in poultry and aquaculture as a feed additive [3]. Its demand has long been met by chemical synthesis. However, owing to the undesirable effects of by-products of synthetic carotenoids, there is a growing interest on natural sources of carotenoids, resulting in renewed focus on microbial sources [4]. *Brevibacterium* sp. KY-4313 [29], *Haloferax alexandrinus* TM^T [2], and *Gordonia jacobaea* MV-1 [40] are some of the microbes with the potential of producing cx on the commercial scale.

Here, we report the characterization of a new cx-producing soil bacterium, K44, and the antioxidant activities of its two cx isomers.

Materials and Methods

Soil Sampling and Isolation of Bacterial Strain

Soil sample was collected from Kargil district of the State of Jammu and Kashmir, India and transported on ice to the laboratory for enrichment and cultivation. Bacteria were isolated by serially diluting 1 g of sediment sample in enrichment broth that consisted of glycerol (5 g/l), K_2HPO_4 (0.5 g/l), KH_2PO_4 (0.5 g/l), $(NH_4)_2SO_4$ (0.4 g/l), $MgCl_2$ (10 mg/l), $ZnSO_4$ (10 mg/l), $FeCl_3$ (10 mg/l), and $CoCl_2$ (10 mg/l). One hundred microliters of the serially diluted samples was plated out on enrichment agar medium and incubated at different temperature for 7 days. Pure cultures were obtained by streak plating of single colonies on nutrient medium [peptic digest of animal tissue (5 g/l), yeast extract (1.5 g/l), beef extract (1.5 g/l), NaCl (5 g/l), pH 7.4; HiMedia Laboratories, India]. The purified culture was maintained on nutrient agar and stored at 4°C or as glycerol medium [20% (v/v)] of cells at −20°C.

Optimization of Growth Temperature and pH

To determine the optimum growth temperature of the bacterium, Erlenmeyer flasks containing 100 ml of nutrient broth were inoculated with a preinoculum ($OD_{660} \approx 1$) corresponding to 1% of the total culture volume and incubated at 10°C, 20°C, 30°C, and 40°C and 150 rpm for 3 days. Growth was monitored by determining absorbance at 660 nm at intervals. The effect of pH on the growth rate of the bacterium was determined in nutrient broth modified by addition of 1 M HCl (for acidic conditions) or 1 M NaOH (for alkaline conditions) over a pH range of 6.0 to 11 (in high pH broths, precipitated salts were removed by filtration prior to use).

Carbon Substrate Utilization Tests

Profiles for utilization of substrates as sole carbon and energy source were performed by the methods of Duckworth *et al*. [12] using basal mineral agar medium [$Na_2HPO_4 \cdot 7H_2O$ (28.5 g/l), KH_2PO_4 (3 g/l), NaCl (8 g/l), $NaNO_3$ (1 g/l), C source 1%, pH 7.6]. Solutions of substrates were made up in deionized water [20% (w/v)], filter sterilized, and added to a final concentration of 1% (w/v). Cells were grown in nutrient broth, spun down after 48 h, resuspended in the same volume of 0.85% (w/v) NaCl, and 10 µl amounts of cell suspension were spotted onto the surface of agar plates containing the test substrates. Plates were read after 7 days at 30°C, comparing substrate-containing plates with control plates without any added carbon source.

Phenotypic and Chemotaxonomic Characterization

Morphological, physiological, and biochemical tests of the organism were performed by the methods of Cappuccino and Sherman [7]. The Gram stain reaction and acid fast staining were carried out using a Gram staining kit and ZN acid fast stain kit, respectively (HiMedia Laboratories, India) according to the supplier's instructions. Antibiotic sensitivity was tested by spreading a light suspension of bacteria in nutrient broth on the surface of nutrient agar and applying commercially available antibiotic sensitivity test discs (HiMedia Laboratories, India). The plates were incubated at 30°C for 48 h. Fatty acid methyl ester (FAME) analysis was performed at Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh, India.

16S rRNA Gene-Based Phylogenetic Analysis

Genomic DNA of the strain was extracted from 10 ml of culture using the method of Bose *et al*. [5]. The 16S rRNA gene was amplified using universal primers fD1 (5'-AGT TTG ATC CTG GCT CA-3') and rP2 (5'-ACG GCT ACC TTG TTA CGA CTT-3') [43]. The PCR-amplified amplicon of about 1,500 bases was further purified using a QIAGEN PCR purification kit (QIAGEN, Germany) and sequenced at The Centre for Genomic Application, New Delhi, India. The nearly full-length nucleotide sequence of the 16S rRNA gene was analyzed using Lasergene, DNASTAR software (Madison, WI, USA). DNA sequence homology searches of the sequence were performed using the BLAST algorithm of NCBI [1]. Evolutionary distance matrices were constructed by the algorithm of Jukes and Cantor, and evolutionary trees for the datasets were inferred from the neighbor-joining method using MEGA ver. 3 software [19]. The stability relationship was assessed by performing bootstrap analysis of neighbor-joining data based on 1,000 replications.

Determination of G + C Content

The G + C content of the DNA of strain K44 was determined by the thermal denaturation method [24] using a Cary 100 UV-Visible spectrophotometer (Varian Inc., USA). *E. coli* DNA was used as the reference standard.

Pigment Extraction

All the procedures were carried out under darkness. Cells were grown in nutrient medium containing 5% NaCl at 30°C for 3 days. Lyophilized cells from 200 L culture were extracted with a chloroform:methanol [1:1 (v/v)] mix. Isolated pigments were dried *in vacuo* using a rotavapor (BUCHI, Switzerland) at 40°C, resuspended in cold acetone, and clarified by centrifugation at 8,000 ×*g* for 10 min at 4°C. Samples were filtered through filter paper and stored at −20°C under nitrogen until analysis.

Colorimetric Determination of Total Carotenoid Content

Total carotenoid content was determined as described by Liaaen-Jensen and Jensen [22] by scanning in the 200 to 800 nm region using a Lambda 35 UV-VIS spectrophotometer (Perkin Elmer, USA).

Separation and Identification of Carotenoid

Total carotenoids were subjected to silica gel (mesh size 230−400) column chromatography, using non-polar hexane as the initial elution solvent. The polarity of the solvent was then increased by adding chloroform (5−100%) to hexane with an increment of 5% and finally by adding methanol (1−20%) to chloroform with an increment of 1%. The fractions were monitored by TLC (thin-layer chromatography). Major red fractions were pooled for further purification. Reverse phase-high performance liquid chromatography (RP-HPLC) analysis of the major red fraction was performed on a Sunfire C18 column (4.6 × 150 mm) using a W 600 controller pump attached to a PDA 2996 Photo Diode Array detector. All spectra were recorded in the range of 190 to 700 nm using Empower Pro Software (Waters, Milford, USA). Elution was made isocratically using solvent system

acetonitrile:methanol (7:3) at a flow rate of 1 ml/min. Astaxanthin, β-carotene (Sigma, St. Louis, USA), and all-*trans*-cx (CaroteNature GmbH, Switzerland) were used as standards. LC-MS/MS analysis of HPLC-purified fractions and the standard were carried out using a Finnigan MAT model LCQ mass spectrometer linked by an APCI interface [15]. The interface conditions were capillary temperature 250°C, vaporizer temperature 450°C, and corona discharge voltage optimized to 5.0 μA. Spectra were analyzed using XCalibur Software (Thermo Fisher Scientific Inc., USA).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Assay

DPPH free-radical scavenging activities of carotenoids were measured according to the method described by Lee *et al*. [20]. Carotenoid isomers dissolved in tetrahydrofuran (10 μM) were added to 200 μM ethanolic DPPH solution in a 96-well plate. After incubating in the dark for 30 min at room temperature, absorbance was recorded at 517 nm using an ELISA reader (Molecular Devices, USA). The antioxidant activity was expressed as % DPPH radical scavenging activity.

Superoxide Radical Scavenging Assay

Total superoxide radical scavenging activity was determined through the combination of riboflavin photoreduction and nitrite formation [9]. Briefly, a reaction mix of 1.4 ml containing 1.11 ml of 50 mM phosphate buffer, pH 7.4, 75 μl of 20 mM L-methionine, 40 μl of 1% (v/v) Triton X-100, 75 μl of 10 mM hydroxylamine HCl, and 100 μl of 100 μM EDTA was taken in test tubes. To the reaction mix, 10 μM of carotenoid was added followed by a brief pre-incubation at 37°C for 5 min, and then 80 μl of 100 μM riboflavin was added to the tube. The tubes were exposed for 10 min to 2 × 20 W fluorescent lamps fitted parallel to each other in an aluminum-coated wooden box. The control tubes contained an equal amount of buffer instead of sample. After exposure to fluorescent lamp, 1 ml of Griess reagent (0.1% naphthalene diamine dihydrochloride, 1% sulfanilamide in 5% H_2SO_4) was added in each tube. The absorbance of the color so formed was measured at 543 nm using a Lambda 35 UV-VIS spectrophotometer (Perkin Elmer, USA). Corrections were made for the background absorbance at 543 nm without sample and riboflavin. The activity was expressed as % inhibition of nitrite formation by 10 μM carotenoid.

Fluorescence Assays for the Detection of Reactive Oxygen Species (ROS) Generated in THP-1 Cells

The monocytic cell line THP-1 obtained from the National Centre for Cell Science, Pune, India was cultured in RPMI 1640 containing 2 mM L-glutamine (HiMedia Laboratories, India) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (Sigma) in a humidified incubator supplied with 5% CO_2 at 37°C, as described earlier [8, 41, 42]. Cells were seeded at a density of 0.5×10^5 per well in a 96-well plate. Cellular differentiation was induced by subjecting cells to 100 ng/ml of phorbol myristate acetate (PMA) for 2 days, followed by preincubation with 10 μM concentration of tetrahydofuran-carotenoid solution for 24 h. After washing with PBS, 10 μM of DCHF-DA (Sigma) was added and incubated for 1 h. Cells were then stimulated with 100 μmol/l of H_2O_2. The fluorescent signals were quantified in a Nanodrop ND-3300 fluorospectrometer at λ_{exc} 480 nm and λ_{em} 525 nm. Data were collected as relative fluorescent units (RFU) and results were expressed as decrease in percentage relative to untreated cells. Cells were also observed under a fluorescence microscope at 40× using excitation sources and filters appropriate for fluorescein isothiocyanate (FITC), and digital images were captured using a digital camera DXM 1200F and ACT-1 software system (Nikon, Japan).

Bacterial and Nucleotide Submission

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain K44 is AY819732. The strain has been deposited at the Microbial Type Culture Collection (MTCC), IMTECH, India under the accession number MTCC 7402.

Statistics

Data are expressed as the means ± SD from at least three independent experiments, unless otherwise indicated. Statistical analysis was done with the Student's t-test. *P*-values <0.05 were considered statistically significant.

RESULTS

Morphology, Biochemical Characteristics, and Antibiotic Sensitivity of K44

Colonies of strain K44 on nutrient agar appeared circular, convex, smooth, and opaque with entire margins (Supplementary Fig. S1). The strain is a Gram-positive, non-spore-forming cocci that germinated into short rods and exhibited snapping division. The strain was oxidase negative and catalase positive. The optimal growth temperature of the strain was found to be 30°C, and the optimum growth pH was 8. The isolate could utilize fructose, sorbitol, glycerol, and hexadecane as carbon source but not adonitol, arabinose, cellobiose, dextrose, dulcitol, galactose, inositol, inulin, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sucrose, trehalose, and xylose. It did not grow on MacConkey agar; did not produce H_2S; was negative for lysine decarboxylase, ornithine decarboxylase activities; and was negative to indole, methyl red, and Voges–Proskauer tests. The isolate showed positive reaction for arginine dihydrolase activity (data not shown). Other phenotypic properties of strain K44 were compared with related members of the genus *Dietzia* and are recorded in Table 1.

The isolate was sensitive to amikacin (10 μg), amoxicillin (10 μg), augmentin (30 μg), bacitracin (10 U), cephalexin (30 μg), cephaloridine (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), cloxacillin (5 μg), co-trimaxazole (25 μg), erythromycin (15 μg), fusidic acid (10 μg), gentamicin (10 μg), kanamycin (30 μg), lincomycin (2 μg), methicillin (5 μg), neomycin (30 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), novobiocin (30 μg), ofloxacin (1 μg), oleandomycin (15 μg), oxy-tetracycline (30 μg), penicillin V (3 μg), penicillin G (10 U), polymyxin B (300 U), rifampicin (2 μg), streptomycin (10 μg), tetracycline (25 μg), ticarcillin (75 μg), tobramycin (10 μg), and vancomycin (30 μg), and resistant to clindamycin

Table 1. Characteristics of *Dietzia* sp. K44 and related strains.

Characteristics	1	2	3	4	5	6	7
Color of colonies	R	O	O-CR	CR	Y	CR	O
Cell shape	c/r	c/r	r	r	r	c/r	c/r
Growth at/in							
Temperature (in °C)	10–45	ND	20–42	5–30	22–45	20–37	28–37
pH	5–11	ND	6–10	7–10	ND	7–10	7–10
NaCl (in %)	0–12	ND	0–10	0–10	ND	Up to 5	Up to 8
Assimilation of							
Glucose	-	+	+	+	+	+	+
Fructose	+	+	+	+	ND	+	+
Sorbitol	+	ND	-	-	-	-	+
Maltose	-	-	-	-	+	-	+
Trehalose	-	ND	ND	-	-	+	+
Citrate	-	ND	+	-	-	-	-
Nitrate reduction	(+)	ND	ND	-	ND	+	+
Hydrolysis of							
Gelatin	-	ND	-	-	-	-	-
Starch	-	ND	+	-	ND	-	+
Casein	-	ND	ND	-	-	-	-
Urea	+	ND	ND	-	+	-	+
G + C content (%)	71.6	73.2	66.1	69.6	72.3	67	ND

1, *Dietzia* sp. K44; 2, *D. maris* IMV 195 [28]; 3, *D. natronolimnaios* 15 LN1 [10]; 4, *D. psychralcaliphila* ILA-1 [38]; 5, *D. cinnamea* IMMIB RIV-399 [37]; 6, *D. kunjamensis* K30-10 [21]; 7, *Dietzia papillomatosis* N1280 [14]; R, red; O, orange; CR, coral red; Y, yellow; c/r, cocci and rods; r, short rods; ND, not determined; +, positive; -, negative; (+), weakly positive.

(2 μg), ceftazidime (30 μg), nalidixic acid (30 μg), sulfamethaxazole (50 μg), and trimethoprim (2. 5 μg).

16S rRNA Gene Sequence and Phylogenetic Profile

A total of 1,431 nucleotides of the 16S rRNA gene were sequenced; the sequence analysis using data from the GenBank revealed that the isolate belongs to the genus *Dietzia,* showing 98% sequence similarity to *D. maris* DSM 43102. Phylogenetic analysis also indicated a close relationship between these strains, the association being supported with the high bootstrapping values separating the strains. The sequence distance and phylogenetic tree constructed on the basis of distance-matrix data suggested it to belong to the genus *Dietzia.* Interestingly, *Dietzia* isolates of human origin were found to form a separate cluster (Fig. 1).

FAME Constituents

GC analysis of methyl ester derivatives of cellular fatty acids of strain K44 and its comparison with two other

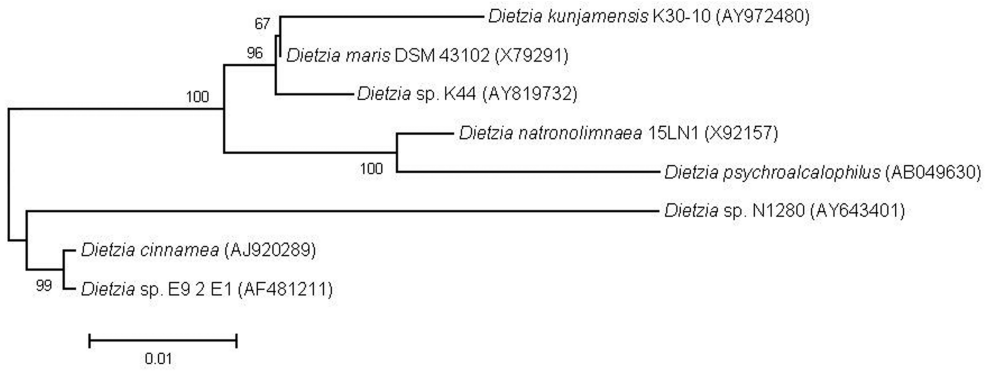

Fig. 1. Phylogenetic tree of reported *Dietzia* species constructed using the neighbor-joining method to show the relationship of *Dietzia* sp. K44 with other members of the genus *Dietzia*.

Numbers at the nodes indicate bootstrap values greater than 65. The tree was derived from the 16S rRNA gene sequence data of the organisms taken from GenBank and accession numbers as given in parentheses.

Table 2. Comparison of FAME analysis data of *Dietzia* sp. K44, *D. maris* IMV 195, and *D. maris* N1015.

Fatty acid type	Name of the fatty acid	Percentage[a]		
		K44[1]	IMV 195[2]	N1015[3]
15: 0	Pentadecanoic acid	0	6	5.7*
Mix of 16:1 w7c and 15 iso 2OH	Delta 6-14-methyl pentadecanoic acid and 13-methyl tetradecanoic acid	12.36	19	1.2*
16: 0	Hexadecanoic acid	20.04	33	36.2*
17:1 w8c	Delta 7-15-methyl-hexadecanoic acid	4.79	15	3*
17:0	Heptadecanoic acid	18.74	6	11.9*
17:0 10 methyl	Delta 7-16-methyl-heptadecanoic acid	1.14	0	7.8*
18:1 w9c	Delta 8-16-methyl-heptadecanoic acid	16.11	18	17.6*
18:0	Octadecanoic acid	8.53	0	2.1*
TSBA 10Me 18:0	Tuberculostearic acid	11.34	12	10.2
19:0	Nonadecanoic acid	6.96	0	0

[a]Composition is given as the percentage of total integrated chromatographic peak areas; [1]this paper; [2][28]; [3][29]; *reported as tetradecanoate, pentadecanoate, hexadecanoate, heptadecanoate, and octadecanoate, respectively

species IMV 195 and N1015 of *D. maris* (Table 2) indicated that *Dietzia* sp. K44 is distinct from the other two strains in terms of their fatty acid composition. Pentadecanoic acid (C 15:0) was absent in K44, whereas in IMV 195 and N1015 it was 6%. Nonadecanoic acid (C19:0) was found to be 7% in K44, whereas this fatty acid was reported to be

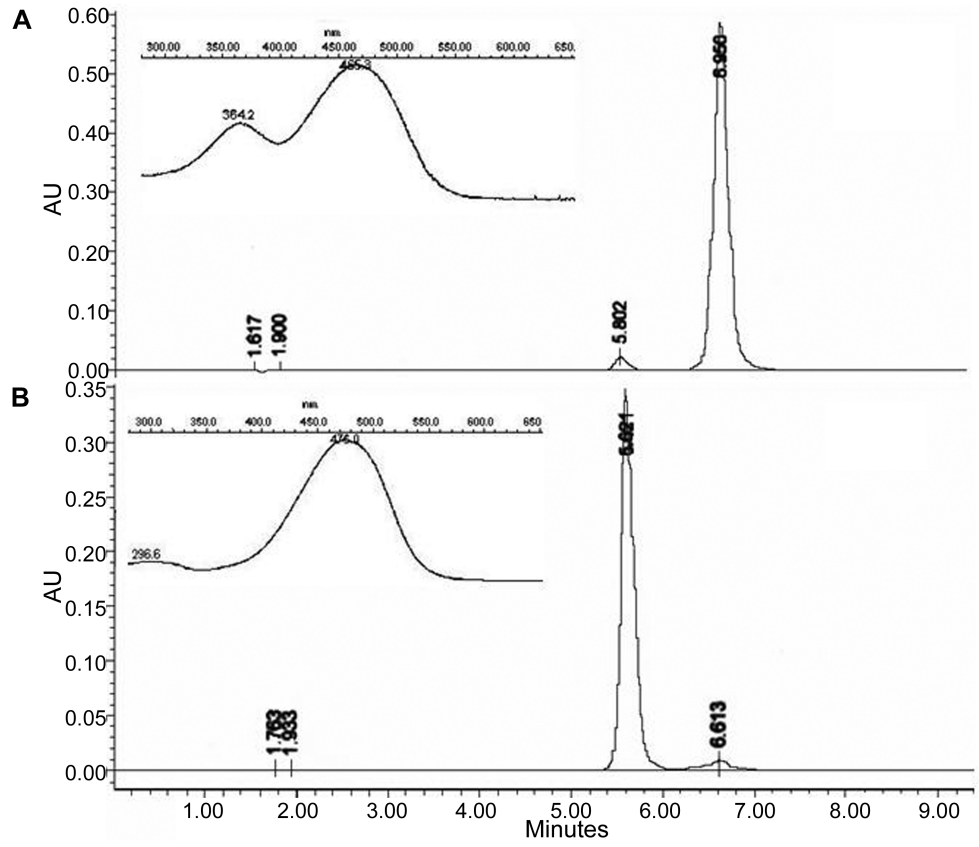

Fig. 2. UV spectra of cx isomers isolated from K44 recorded online during RP-HPLC elution, with different retention times, using a photodiode array detector.
(**A**) *cis*-cx having UV absorption peak at 465.3 and 364.2 nm. (**B**) All-*trans*-cx with single UV absorption peak at 475 nm. Numbers at the top of RP-HPLC peaks indicate the retention time in minutes.

absent in both IMV 195 and N1015. Heptadecanoic acid (C17:0) and octadecanoic acid (C18:0) in K44 were higher in comparison with the other two strains.

All-*trans*-cx and 9-*cis*-cx Isomers in K44

The total carotenoid content was 231 µg/g dry weight. The major pigment from K44 eluted as a red band in silica gel column chromatography, and on further purification by RP-HPLC resolved into two peaks having λ_{max} 475 nm and 465.3 (364.2) nm, respectively. When an equimolar mixture of the peak 1 (Fig. 2B) and authentic cx was loaded onto HPLC, they were found to co-elute. These two peaks (Fig. 2A, 2B) were isolated in preparative scale for mass spectrometric analysis. The molecular mass (both 564 Da) as well as fragmentation patterns of the two peaks were found to be the same on LC-MS/MS analysis and coincided with the standard all-*trans*-cx (Supplementary Fig. S2, S3, and S4). The base peak at *m/z* 565 is a protonated molecule $[M+H]^+$. The molecular radical ion $[M]^{\bullet+}$ having the mass *m/z* 564 appeared with high intensity when observed with other easily ionized compounds. The other ion recorded at *m/z* 547 was identified as $[MH-18]^{\bullet+}$ and this was formed by free-radical fragmentation from the radical cation $[M+H]^{\bullet+}$. These two peaks were identified as all-*trans*-cx and 9-*cis*-cx, respectively.

Antioxidant Activities of the All-*trans*-cx and 9-*cis*-cx Isomers

Both cx isomers exhibited 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging activities. The *cis*-isomer was found to have a 1.8-fold higher free-radical scavenging activity than that of all-*trans*-isomers (Fig. 3A).

Cx isomers also showed scavenging activity against superoxide radicals generated from irradiation of riboflavin when measured indirectly as % inhibition of nitrite production. The *cis*-isomer was found to be 1.4-fold more

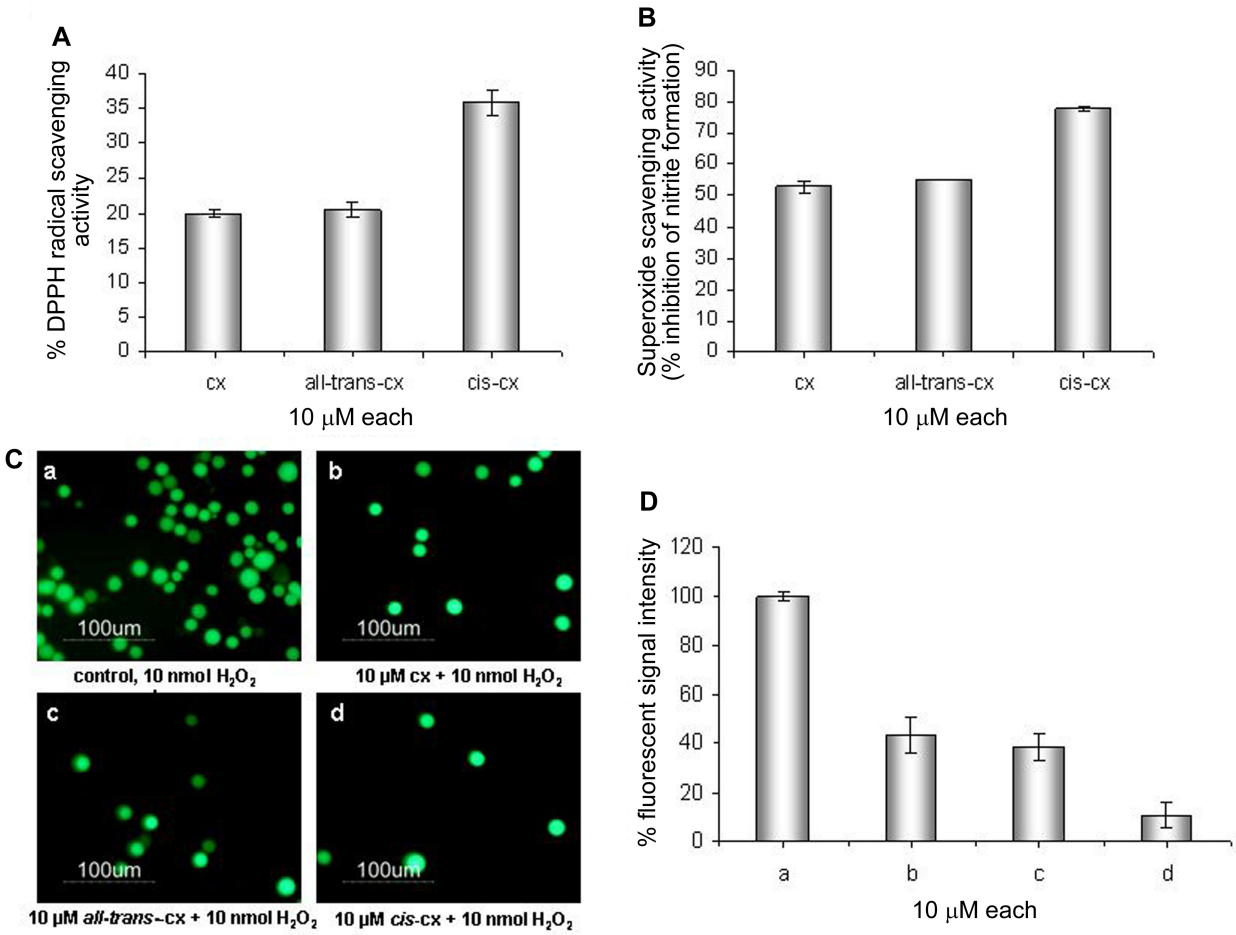

Fig. 3. Antioxidant activity of cx isomers.

DPPH radical scavenging activity (**A**); superoxide scavenging activity measured as % inhibition of nitrite formation (**B**); Inhibition of ROS generation in THP-1 cells by cx isomers measured by fluorescence microscopy, (**C**) with THP-1 cells, a (H_2O_2 alone), b (cx standard + H_2O_2), c (all-*trans*-cx + H_2O_2), and d (*cis*-cx+ H_2O_2), respectively; and Quantitations of fluorescence signaling (**C** a, b, c, d) in THP-1 cells (**D**). Experimental details are as given in Materials and Methods. Values shown are the means ± SD from three experiments. p Values are <0.05.

active than the all-*trans* form (Fig. 3B). The *cis*-cx was also found to be more effective in inhibiting the generation of reactive oxygen species (ROS) in THP1 macrophages on stimulation with H_2O_2 (Fig. 3C, 3D).

DISCUSSION

The present study reports isolation of a bacterium, designated K44, from the loamy soils of Kargil, India rich in phosphorous and potassium (data not shown). It was observed that K44 grew as smooth red colonies on nutrient agar plates after incubation of 3 to 5 days at 30°C. Physiological, biochemical, and chemotaxonomic properties and 16S rRNA gene sequence profile of K44 showed their similarity to *D. maris* DSM 43102. The strain K44 differs from *D. maris* in utilization of glucose and sorbitol as carbon sources, antibiotic susceptibility pattern, G + C content, as well as in the percentages of components of methyl ester derivatives of cellular fatty acids, and thus indicated it to be distinct from *D. maris* DSM 43102.

The 16S rRNA gene sequence comparison of the isolate K44 clearly showed that it is a member of the family Dietziaceae and it contained the entire eleven signature sequences of this family [45]. Phylogenetically, the strain K44 clusters in the nonpathogenic environmental *Dietzia* strains and not in the three *Dietzia* strains of human origin. Three strains of human origin have been identified as *Dietzia* strain X, an etiological agent of confluent and reticulated papillomatosis [28], *D. cinnamea* isolated from a perianal swab of a patient with a bone marrow transplant [45], and *Dietzia* sp. E9_2E1 associated with human endodontic infection [27]. Comparison of the 16S rRNA gene sequences of these strains shows variation in the first signature nucleotide sequence of the genus *Dietzia* from 5'-UAA GGC CCU UUC GGG GGU ACA-3' to 5'- UAA GGC CCU UC- -GG GGU ACA-3'.

A study on the pigments of the genus *Rhodococcus*, the genus to which *D. maris* originally belonged, has reported for the presence of β-carotene and unidentified carotenoids (xanthophylls) in *R. maris* [16]. A different strain of the genus, *Dietzia* sp. CQ4, has also been reported to accumulate cx and echinenone along with c.p. 450 monoglucoside [37]. However, percentages of carotenoids in the total extract were found to vary with respect to K44.

As the fragmentation patterns of *cis/trans* isomers (Supplementary Fig. S1, S2, S3) are identical, structural elucidation using mass spectrometry was not sufficient. Absorption spectra of cx isomers were recorded at the chromatographic peak maximum in LC-MS/MS analysis. The appearance of a *cis*-band in most carotenoids leads to a hypsochromic shift of λ_{max} compared with the λ_{max} of the all-*trans* molecule, with a characteristic additional band at around 340 nm [10]. Thus, the major pigment being produced by the bacterium was identified as cx isomers. The individual pure fractions of these isomers were found to undergo isomerization on storage. It is well known that carotenoids are highly reactive molecules, the polyene backbone being the cause of instability including their susceptibility to oxidation. They predominantly occur in the thermodynamically stable all-*trans* configuration, as the presence of a *cis* double bond creates greater steric hindrance between nearby hydrogen atoms and/or methyl groups, thereby decreasing the stability [6]. Geometric isomerization of *trans*-carotenoids to the *cis*-form is known to be promoted by heat, light, and acids [13].

In the field of antioxidants, isolation and identification of antioxidants from bacterial strains inhabiting extreme environments has been widely reported [11, 31, 33]. Many studies, both *in vitro* and *in vivo*, have reported cx to possess higher antioxidant activity than other carotenoids [38, 47]. Furthermore, our earlier findings predicted the role of 9-*cis*-cx and all-*trans*-cx in inducing apoptosis in THP-1 cells [41]. Following these findings, in this work, we further studied the effect of isomerism on the active oxygen species scavenging ability of cx isomers. Macrophages synthesize toxic oxygen metabolites when the cells are stimulated by a variety of soluble compounds [47]. It was observed that the 9-*cis*-cx was more active in the suppression of ROS generation *in situ* than the all-*trans*-isomer. The 9-*cis*-cx was also found to have higher antioxidant activity compared with the all-*trans*-cx, as evidenced by free-radical and superoxide radical scavenging assays. Like higher antioxidant activities of *cis*-isomers of β-carotene and astaxanthin than their all-*trans*-isomers [21, 23], the higher antioxidant activity of the *cis*-cx than the all-*trans*-cx was also observed. It has been postulated that the *cis* form possesses a higher potential energy, which increases its susceptibility to various reactions and hence a higher antioxidant activity than its all-*trans* isomer. It has also been suggested that structural parameters besides the length of polyene chain might contribute to the antioxidant properties of carotenoids.

Canthaxanthin is used widely as an approved food colorant and feed additive. However, little importance has been given to bacteria as potential carotenoid producers as they frequently do not produce carotenoids of interest in sufficient quantities. Nonetheless, their unicellular nature and higher growth are suitable for large-scale production in fermenters [34]. Exposure to high salt concentrations usually results in cessation of growth in all organisms; however, carotenoid production in *Dietzia* sp. K44 was found to increase with increase in salt concentration in the medium. Further studies on media optimization and/or carotenoid mutants need to be done to improve the canthaxanthin content of the bacterium to economically viable values.

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