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Research Article

Halorubrum sp. TBZ112, an Extremely Halophilic Carotenoid-Producing Archaeon Isolated from Urmia Lake

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ABSTRACT

Background: Carotenoids are organic pigments with substantial applications in neutraceuticals, pharmaceuticals, cosmetics and food industries. Considering the importance of carotenoids, we aimed to isolate and identify a carotenoid-producing microorganism in the present study.
Methods: Gram-negative, aerobic and rod-shaped archaeon, *Halorubrum* sp. TBZ112 (KCTC 4203 and IBRC-M 10773) producing carotenoids with circle and red colonies were isolated from Urmia Lake in Northwest Iran. Phenotypic characterization and molecular identification of isolate were also conducted. In order to study the ability of TBZ112 to produce carotenoids as well as the production profile, carotenoids were extracted using acetone-methanol solution (7:3 v/v). Then, the carotenoid content of the extract was evaluated using UV spectroscopy and thin-layer chromatography (TLC). The carotenoid profile was analyzed using liquid chromatography-mass spectrometry (LC-MS) techniques.
Results: The 16S rRNA analysis showed that TBZ112 had the highest similarity with *Halorubrum chaoviator* Halo-G*^{T} (99.78%). The total carotenoid content of strain TBZ112 was found to be 11.7 mg/l. The LC-MS analytical results indicated that carotenoids that were produced included bacterioruberin, lycopene and β-carotene. Among them, bacterioruberin was predominant.
Conclusion: Consequently, we can suggest that future studies should investigate this new and natural source for producing carotenoids.

Introduction

Carotenoids are yellow, orange, or red colored pigments produced by non-phototrophic procaryotes to higher plants.[1,2] Regarding their colorant and anti-oxidant properties, carotenoids are utilized in the cosmetics, food, and feed industries.[3] Moreover, carotenoids may be used as agents for protection against the effects of the sun. This proposal was based on the fact that they protect their organism against reactive oxygen species (ROS) and UV radiation from the sun by absorbing light in the 350–500-nm range.[4] This is within the range that should be added to obtain broad-spectrum protection, and, therefore, carotenoids that absorb in this range (e.g., bacterioruberin) would provide complementary protection, increasing protection from radiation above 400 nm.

Even though synthetic methods produce carotenoids with high purity and little fee, the synthetic carotenoids often contain a number of reaction precursors or non-biological by-products that possibly could produce undesired side effects. Consequently, they are not eco-friendly. Biological production of carotenoids have many advantages especially presence of wide range of their potential natural sources that have the ability to produce naturally-occurring stereoisomers. The production of carotenoids using microorganisms might be beneficial compared to macroalgae, plants, and chemical-production methods because microorganisms are unicellular, have a high growth rate, can be produced irrespective of the season and

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geographical conditions, are controllable and have predictable yields, and are safe to use.[5,6] Many carotenoid-producing micro-organisms have been isolated from numerous extreme ecosystems, such as excessive salinity, intense light as well as acidic, alkaline, and thermophilic habitats.[5,7]

Most halophilic archaea in the family of *Halobacteriaceae* are bright red or orange in colour due to their red membrane that consists of carotenoids. The main carotenoids of the halophilic archaea are C_{50} carotenoids, mostly bacterioruberin and its derivatives, including monoanhydrobacterioruberin and bisanhydrobacterioruberin.[8,9] Although several reports have addressed the carotenoid profile of eubacteria, only a few papers have described carotenoid production from archaea.[10] Kelly et al. proposed that bacterioruberin is a typical carotenoid found in halophilic bacteria and archaea.[8] Kushwaha et al. determined numerous strains of halophilic archaea-produced bacterioruberin and monoanhydrobacterioruberin, including *Halobacterium salinarum*, *Halobacterium saccharovorum*, *Halobacterium vallismortis*, and other haloarchaea.[9] Moreover, bacterioruberin has also been found in a highly radiation-resistant bacterium, *Rubrobacter radiotolerans*.[11]

The OH scavenging impact of bacterioruberin was examined using a system of thymine degradation and compared with those of cysteine and β-carotene. Bacterioruberin (which has 13 conjugated double bonds) scavenges hydroxyl radicals much more effectively than β-carotene.[11] In addition, it also has been demonstrated that bacterioruberin provides resistance against oxidative, DNA-damaging agents in vivo.[11]

With the exception of our previous study reported recently,[6] to the best of our knowledge, there are two articles that describe carotenoid production from a *Halorubrum* strain.[12,13] Urmia Lake is the largest brine lake in the Middle East and the second-largest saltwater lake in the earth.[14] Even so, very few studies of the lake have been reported, and no study was published on the carotenoid-producing prokaryotics that live in this hyper-saline lake. Accordingly, following our previous report in which we described the isolation and description of some halophilic bacteria from Urmia Lake,[15] we herein report the isolation and characterization of an extremely halophilic archaeon from Urmia Lake and elucidate its potential for use in the production of carotenoid compounds.

Materials and Methods

Chemicals and reagents

The β-carotene (CAS No. 7235-40-7) and lycopene (CAS No. 502-65-8) standards were obtained from Sigma–Aldrich (Steinheim, Germany). β-carotene was stored at -20 ºC and lycopene at -70 ºC. Ammonium acetate, triethylamine, and ascorbic acid (> 95%) were purchased from Merck Chemicals (Darmstadt, Germany). All HPLC gradient grade solvents were purchased from Caledon Laboratories, Ltd. (Ontario, Canada).

The contents (per liter) of the marine agar medium[16] were $MgCl_2.7H_2O$, 8.8 g; Na_2SO_4, 3.24 g; $CaCl_2$, 1.8 g; KCl, 0.55 g; $NaHCO_3$, 0.16 g; KBr, 0.08 g; $SrCl_2$, 34.0 mg; H_3BO_3, 22.0 mg; Na_2O_3Si, 4.0 mg; NaF, 2.4 mg; NH_4NO_3,1.6 mg; Na_2HPO_4, 8.0 mg; peptone 5 g; yeast extract 1 g; agar 15.0 g, and various concentrations of NaCl from zero to the saturation level, i.e., > 30% w/v). The pH of the medium was adjusted between 7 and 10 in advance autoclaving.

The contents (per liter) of the marine broth[16] were $MgCl_2.7H_2O$, 5.9 g; $MgSO_4$, 3.24 g; $CaCl_2$, 1.8 g; KCl, 0.55 g; $NaHCO_3$, 0.16 g; KBr, 0.08 g; $SrCl_2$, 34.0 mg; H_3BO_3, 22.0 mg; Na_2O_3Si, 4.0 mg; NaF, 2.4 mg; NH_4NO_3, 1.6 mg; Na_2HPO_4, 8.0 mg; peptone 5 g; yeast extract, 1 g; and various concentrations of NaCl from zero to the saturation level, i.e., > 30% w/v). The pH of the medium was adjusted between 7 and 10 in advance autoclaving.

Culture and isolation

Water samples collected from Urmia Lake were inoculated on marine agar medium with various NaCl concentrations, pH values between 7 and 10, and incubated at temperatures in the range of 15 to 50 °C. For the isolation of pure cultures, single colonies were picked up from the plates and used for Gram staining and stock preparation and they were grown in marine broth with agitation at 120 rpm in an orbital shaker (Shaking Incubator VS-8480, Korea) in the dark for 9 days. The archaeal stock was prepared in the marine broth with glycerol (70:30 v/v) and stored at -70 ºC for later use.

Extraction of genomic DNA and sequencing of 16S rRNA

DNA was extracted using a modified version of the Corbin 'Genomic DNA isolation' protocol.[17] Briefly, cells were lysed by freeze-thaw cycles in liquid nitrogen and then suspended in solution I [Tris 10 mM (pH 7.4), EDTA 1 mM, sodium dodecyl sulfate (SDS) 0.5%, proteinase K 0.1 mg/ml] and lysed by incubation at 37 °C for 1 hour. Then, solution II [0.8 M NaCl and 1% CTAB] was added to the lysates and incubated at 65 °C for 20 min, and, finally, genomic DNA was extracted with an equal volume of chloroform-isoamylalcohol (24:1 v/v). Nucleic acids were precipitated from the aqueous phase with 0.6 volume of isopropanol.

The 16S rRNA gene was amplified using the polymerase chain reaction (PCR) with two primers, 20F (5´ -TCC GGT TGA TCC TGC CG- 3´)[18] and

1530R (5´ -AGG AGG TGA TCC AAC CGC A-3´).[19] The PCR was performed using a thermal cycler (Eppendorf International) with a 50-µl reaction containing 1.5 µl of MgCl2, 1 µl of each dNTP, 0.5 µl of each primer, 5 µl of PCR buffer, 36.5 µl of H2O, and 1 µl (5 U) of Taq DNA polymerase (Cinnagen, Iran). Initial denaturation was conducted for 3 min at 95 °C. It was followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 45 °C for 40 sec, and extension at 72 °C for 1.5 min with a further 10-min extension at 72 °C. The amplified DNA fragment was separated using 1% agarose gel electrophoresis, and, then, the DNA fragment was extracted from the gel and sequenced by Faza Biotech Co. The sequence was compared with reference 16S rRNA gene sequences available in the NCBI GenBank database BLAST using blastn and megablast software and the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net).[20] Sequence alignment was conducted using the multiple sequence alignment program CLUSTALX (version 1.83)[21] Then, relative type strains of TBZ112 were chosen for a detailed phylogenetic analysis, and, finally, phylogenetic trees were constructed using the neighbour-joining (NJ) and maximum-likelihood (ML) methods in the MEGA version 5 software package.[22]

Physiological characterization of the isolate

Gram-staining was performed as described by Gerhardt et al.[23] and confirmed by Dussault.[24]To investigate the basic physiological characteristics of the isolate, the MacFaddin[25] and Barrow et al.[26] methods were used for the following tests: Oxidase and catalase reactions, phenylalanine deaminase, nitrate reduction, hydrolysis of urea, gelatin, starch, Tween 80 and tyrsosin, H_2S and indol production from L-cysteine and tryptophan. To examine nitrate reduction, 0.2% (w/v) KNO_3 was added to the liquid media. Gelatin hydrolysis and starch hydrolysis were tested by flooding cultures on solid media containing 1% (w/v) gelatin and starch respectively. Hydrolysis of Tween 80 was tested on solid media supplemented with 1% (w/v) Tween 80. Tyrosine hydrolysis was evaluated by the appearance of a clear zone on marine agar medium culture containing 5 g/l tyrosine. H_2S production was tested in liquid media supplemented with 0.01% (w/v) of L-cysteine. The indicator used in this experiment was a band of paper impregnated with lead acetate placed in the neck of the tube.

Extraction and analysis of pigments

For bio-production of carotenoids, the cells were cultured in marine broth (NaCl 15–30% w/v, pH 7–10) in the orbital shaker and agitation at 120 rpm for 9 days at 30 °C in a non-illuminated environment. To extract carotenoids, 100 ml of the marine broth cultures were centrifuged at 8,000 rpm for 10 min at 4 ºC. The supernatant was separated and a mixture of acetone-methanol (7:3 v/v)[27] containing butylhydroxytoluene (BHT) (0.1% as antioxidant) was added to the pellet. Then, the pelleted cells were frozen and thawed using liquid nitrogen to facilitate extraction, and this was followed by centrifugation at 10,000 × g for 10 min at 4 °C. Successive extractions were conducted until both the solvent and the cells were colourless. The solvent was evaporated under a stream of nitrogen and the pigments were dissolved in 10 ml of acetone (containing 0.1% BHT). The samples were wrapped with aluminium foil to protect them from light. The extracts were stored under nitrogen at -70 °C. The extraction methods and analyses were performed in dark environments.

UV-Visible Spectroscopy

UV-VIS spectra of the extraction solution were recorded at 200–700 nm using a spectrophotometer (Shimadzu UV-1800 Series, Kyoto, Japan). The total amount of carotenoid was calculated by measuring the optical density of the sample in 495 (λmax of our extraction solution). The total amount of carotenoids was estimated as described previously.[6]

Thin-Layer Chromatography

Thin-layer chromatography (TLC) was used to confirm the presence of carotenoid pigments in the extract. For this analysis, the acetone extract was placed on a TLC silica gel GF254 plate (Merck, Darmstadt, Germany) and developed in hexane:acetone (7:3). After development, the individual spots were distinguished visually and sprayed with a saturated solution of antimony pentachloride ($SbCl_5$) in chloroform (1:10 v/v).[6]

Liquid chromatography–mass spectrometry (LC-MS)

The extracted solution was centrifuged and the supernatant was filtered through cellulose acetate filters (25 mm, 0.45 mm; VWR International). Samples were held on ice and covered with aluminium foil to decrease isomerization and oxidation of the carotenoids by light irradiation. Chromatographic separation was done on an Agilent 1200 series HPLC system, including a quaternary pump and a degasser equipped with a G1315B Diode Array Detector. The accompanying Agilent LC Chemstation was used for instrument control, data achievement and data processing. HPLC analysis was performed using a Eurosphere RP-column (100-5 C18 column, 300×4.6 mm Knauer, Germany) by isocratic elution with a flow rate of 0.8 ml/ min. The mobile phase was acetonitrile-dichloromethane-methanol (70:20:10 v/v/v), 20 mM ammonium acetate and 0.1%

triethylamine. The temperature was retained at 20 °C, and UV detection was performed at 450 nm. Both the extracts and standards were injected (injection volume: 20 µl) into the reverse-phase column and all of the trans-isomers were identified using the comparison of retention times and comparison of the UV spectra of the extracts with a standard mixture. The experiment was run in triplicate. The mass spectra were recorded in the positive-ion mode in the mass range from 300 to 2000 m/z. The mass spectrometer parameters were set as follows: Nebulizer pressure was 40 psi, drying gas flow was 20 l/min, and gas temperature was 250 °C. The capillary voltage was 5000 V. Ions were monitored in the scan mode. The identification of carotenoids was performed by comparing retention time, UV spectra, and the characteristics of the mass spectra (protonated molecule ([M+H]+) and its MS/MS fragments. All of the carotenoids were monitored at 450 nm with a UV-visible detector.

Results

Morphologic and genotypic characteristics

Cells were Gram-negative and rod-shaped. Strain TBZ112 formed circular and red colonies on marine agar. Strain TBZ112 didn't grow in the absence of NaCl and could tolerate NaCl up to the saturation level (> 30% w/v) at 30 °C. It did not grow in acidic medium but tolerated alkaline medium (pH 10). Strain TBZ112 grew at 15 °C (very weak) and tolerated higher temperatures up to 50 °C. The 16S rRNA gene sequence analysis revealed that strain TBZ112 had 99.78% similarity with Halorubrum chaoviator Halo-G*^{T}.[28] Figure 1 shows the phylogenetic tree of TBZ112 constructed using the neighbour-joining (NJ) method. Analysis showed that strain TBZ112 fell within the Halorubrum cluster and made a separate cluster with *Halorubrum chaviator* with which it had the highest 16S rRNA sequence similarity. The same classification was obtained using the maximum-likelihood (ML) method (tree not shown). The 16S rRNA gene sequence of TBZ112 was submitted to the GenBank under accession number KF152924.1. TBZ112 was deposited in the Korean Collection for Type Cultures (KCTC) under the code of 'KCTC-4203' and also in the Iranian Biological Resource Center (IBRC) under the code of 'IBRC-M 10773' for public accession. According to the phenotypic and genotypic characterizations, TBZ112 is a mesophilic and extremely halophilic archaeon.

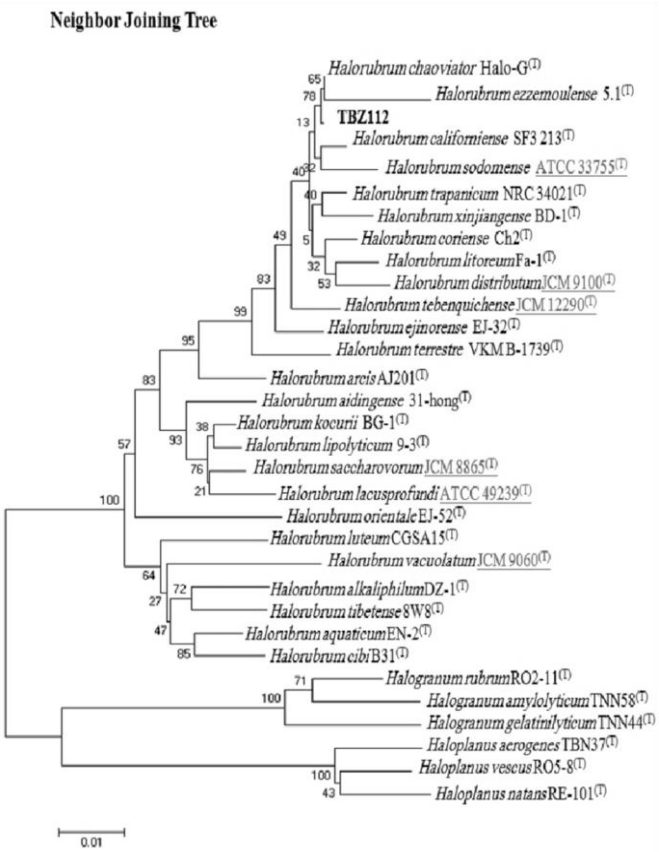

Figure 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain TBZ112. The sequence alignment was performed using the CLUSTALX program and the tree was generated using the neighbor-joining method in MEGA 5. Bootstrap values are indicated on the branches. The scale bar indicates 0.01 changes per nucleotide position.

Table 1. Differential characteristics of strain TBZ112 and type strains of related *Halorubrum chaoviator* (Halo-G*^{T}, AUS-1 and Naxos II).

Feature	TBZ112	Halo-G*^{T}	AUS-1	Naxos II
Morphology	Rod	Rod	Rod	Rod
Gram staining	-	-	-	-
Pigmentation	Red	Red	Red	Red
Growth at 15-30% NaCl	+	+	+	+
Growth at NaCl > 30%	+	-	-	-
Growth at pH > 8.5	+	-	-	-
Nitrate reduction	-	-	+	-
Starch Hydrolysis	+	+	-	+
Gelatin Hydrolysis	+	-	-	-
Tween 80 Hydrolysis	-	-	-	-

Physiological and biochemical characterization

The catalase test was positive, and the oxidase test was negative. Nitrate was not reduced to nitrite, and indole was produced. The hydrolysis of gelatin and starch was found to be positive, whereas Tween 80 was not hydrolyzed. Urease activity was found to be negative. Phenylalanine deaminase was negative. H2S formation was positive. The differential characteristics of strain TBZ112 and type strains of related Halorubrum species are summarized in Table 1.

UV- Visible spectrum of the extract and total carotenoid content

A fine extraction method should release all the carotenoids and pass them into the extracting solvents without changing their structures. To select the suitable solvent, acetone, methanol, hexane, and acetone/methanol (7:3 v/v) were examined individually and the best recovery was obtained using acetone/methanol (7:3 v/v). The pigment solutions in the acetone/methanol mixture showed the characteristic absorptions of carotenoids (Figure 2).[29]

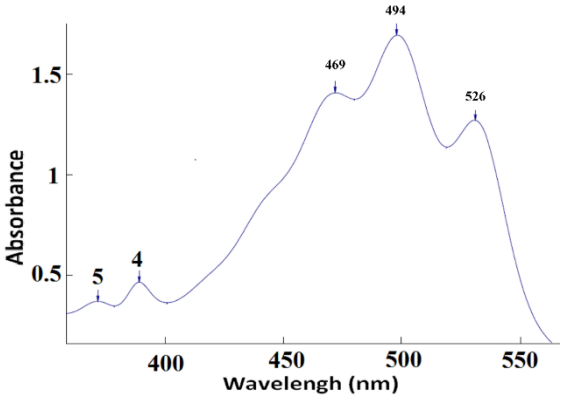

Figure 2. UV-VIS spectrum of acetone extract from strain TBZ112.

Britton et al. indicated that bacterioruberin and its derivatives displayed the distinctive spectral peaks of red carotenoids at approximately identical absorption maxima at 467, 493, and 527 nm for the three-fingered peaks and at 370 and 385 nm for two cis peaks.[29] Figure 2 shows that the pigments in the extract solution had absorption peaks at 469, 494, and 526 nm, indicating that bacterioruberin was the main component in the extracted sample. The total carotenoid content of strain TBZ112 grown in the marine broth medium (NaCl 25%, pH 7.5, and temperature 30 ºC) that incubated in the orbital shaker at 120 rpm for 9 days, was found to be 11.7 mg/l.

Thin-Layer Chromatography of the carotenoid extract

After progress of the TLC test, five spots were detectable that were blue after staining the plate with antimony pentachloride ($SbCl_5$) in chloroform,[30] verifying the existence of carotenoids in the extraction solution. It is worth mentioning that, at low concentrations of the extract, in spite of visualizing the spots, only one spot was stained blue with $SbCl_5$.

Liquid chromatography–mass spectrometry (LC-MS) of the carotenoid extract

Having confirmed that carotenoids were produced by strain TBZ112 using UV-VIS spectroscopy and TLC, LC-MS analysis was conducted to identify the profiles of the carotenoids that were produced. The peaks of all trans-isomers of lycopene and β-carotene were recognized by comparing the retention times of authentic standards and the UV spectra. Since most of the major cis-isomers of the carotenoids are not available in the market, they were identified by their UV absorption characteristics and also by comparing the chromatograms obtained with the fully investigated isomers of lycopene and β-carotene reported by Müller et al.[31] Bacterioruberin was characterized by comparing the UV and mass spectra with those reported in the literature.[8,32]

HPLC analysis of TBZ112 carotenoids showed five distinctive peaks that were identified as bacterioruberin (peak 1), all-trans-lycopene (peak 2), 13-cis-lycopene (peak 3), all-trans-β-carotene

(peak 4), and all-cis-β-carotene (peak 5) (Figure 3). As illustrated in Figure 3, the present reverse-phase isocratic HPLC method was used to separate three main carotenoids with good resolution and in a short time (14 min). The retention times of lycopene and β-carotene in the sample were in accordance with genuine standards.

The UV–VIS spectra of the carotenoids also served as a useful source for their detection and the DAD allowed the UV-VIS spectrum of each component to be determined online. Each carotenoid had distinctive features, including wavelength of maximum absorption (λmax) and the shape of its spectrum was unique. The maximum absorption wavelengths for the carotenoids of interest, detected by the diode array detector, were 466, 495, and 528 nm for bacterioruberin; 448, 474, and 505 nm for lycopene; and 425, 455, and 482 nm for β-carotene. Since the ionization mode was positive, most of the m/z data were [M+H]+ and the mass data of the compounds identified are given in Table 2 and the supplementary Figure 1. Derived from the mass fragmentation interpretation, the compounds with pseudo molecular ionic peaks at m/z 444 and 537 for the [M+H]+ may be identified as lycopene and β-carotene while fragmented ionic peaks at m/z 683.2, 705.2, 723.2 and 741.2 may be identified as bacterioruberin.

Discussion

Carotenoids have considerable commercial interests and are applied as coloring agents in pharmaceuticals, cosmetics and food industries.[33] There has been an increasing attention towards natural sources of carotenoids.[34,35]

Bacterioruberin and its derivatives produced by halophiles have immense biological effects such as scavenging the hydroxyl free-radicals formed by radiography, UV irradiation and H_2O_2 exposure. Very few studies of bacterioruberin activities like immunemodulators and prophylactic agents against cancers have been published.[36,37] Marshall et al. mentioned that bacterioruberin is a ubiquitous and plentiful pinkish-red pigment present moderately or abundantly in halophilic archaea. They employed the Resonance Raman spectroscopy method to identify biomarkers derived from a variety of halophilic archaea, including *Halobacterium salinarum* NRC-1, *Halococcus morrhuae*, and *Natrinema pallidum* that adapted to hypersaline environments.[38]

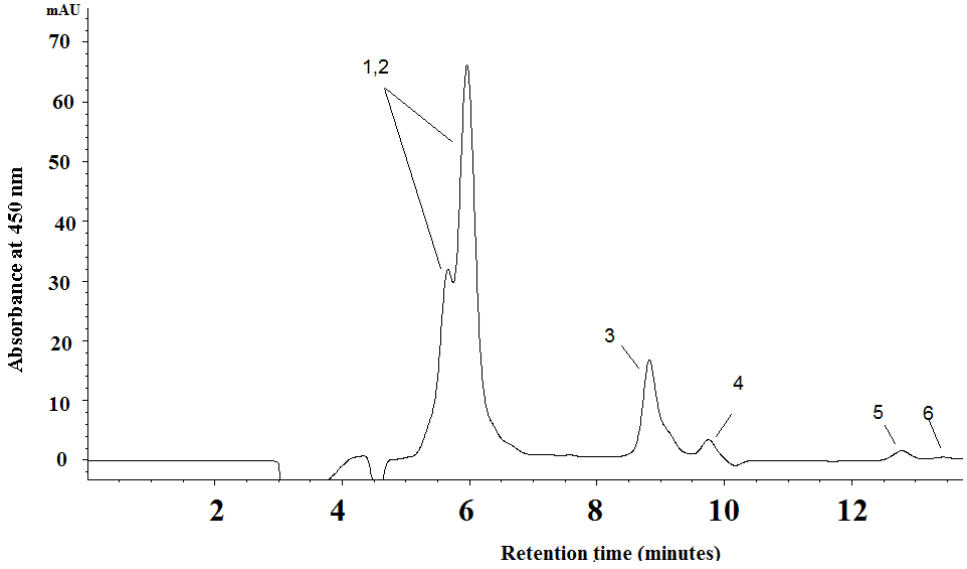

Figure 3. Reverse-phase liquid chromatography of the major carotenoids of strain TBZ112. Column: 100-5 C18 column (300 by 4.6 mm, Knauer, Germany). Eluent: acetonitrile-dichloromethane-methanol (70:20:10, vol/vol/vol). Flow rate: 0.8 ml/min. Detection: 450 nm. Peak identities: peak 1, bacterioruberin; peak 2, all-trans lycopenes; peaks 3, 13-cis-lycopene; peak 4, all-trans- β-carotene; peak 5, all-cis- β-carotenes.

Table 2. Identification of carotenoids extract from strain TBZ112 elucidated by ESI ion mode showing their molecular masses and molecular formulas.

Carotenoid	Molecular formula	Approximate molecular mass (Dalton)	Fragmentation mass (m/z)	MS/MS fragment ion (m/z)
Bacterioruberin	$C_{50}H_{76}O_4$	740	741	723 $[M+H-18]^+$ 705 $[M+H-18-18]^+$ 683 $[M+H-58]^+$
Lycopene	$C_{40}H_{56}$	536	537	444 $[M+H-92]^+$
β-carotene	$C_{40}H_{56}$	536	537	444 $[M+H-92]^+$

Asker et al. studied the production of carotenoids from *Haloferax alexandrinus* strain TMT. The carotenoids detected by this extremely halophilic archaeon using HPLC on a silica gel column were β-carotene, 3-Hydroxyechinenone, Canthaxanthin, trisanhydro-bacterioruberin, monoanhydro-bacterioruberin, bacterioruberin isomers and bacterioruberin.[7,39] Ronnekleiv and colleagues reported that *Heloferax vokanii* contained (2S,2′S)-bacterioruberin, monoanydrobacterioruberin, (2S,2′S)-bisanhydrobacterioruberin,3,4-dihydromonoanhydrobacterioruberin and two undecaene $C_{50}H_{74}O_4$ carotenoids, the C_{45}-carotenoid (2S)-2-isopentenyl-3,4-dehydrorhodopin and lycopene.[32] Mandelli et al. investigated carotenoid production by the extremophile microorganisms *Halococcus morrhuae*, *Halobacterium salinarium* and *Thermus filiformis*. The major carotenoid was all-trans-bacterioruberin in *Halococcus morrhuae* and *Halobacterium salinarium*.[40] Asker and Ohta found that the total carotenoid production by *Haloferax alexandrinus* strain TMT, an extremely halophilic archaeon, was about 6.34 mg/l respectively.[41] Abbes et al. reported that carotenoid production yields of the Haloarchaea isolated from the solar saltern of Sfax, Tunisia ranged between 5.66 and 7.63 mg/l in which bacterioruberin was the main component.[42]

Strain TBZ112 (KCTC 4203 and IBRC-M 10773), a Gram-negative, aerobic, rod-shaped and mesophilic and extremely halophilic archaeon was isolated from Urmia Lake. TBZ112 did not grow in the absence of NaCl and tolerated the salt up to saturation level. It could tolerate higher temperatures up to 50 °C and alkaline media; pH 10. 16S rRNA gene sequence revealed that TBZ12 has the highest similarity of 99.78% with *Halorubrum chaoviator* Halo-G^{*T} (AM048786). Accordingly, TBZ112 is considered a mesophile and extremely halophilic archaeon related to *Halorubrum*-type stains strains. Carotenoid extraction was carried out using acetone-methanol (7:3 v/v). UV-VIS spectroscopy results showed the characteristic absorptions of carotenoids. This result was further confirmed by TLC with $SbCl_5$. In recent years, the most appropriate technique for the investigation of carotenoids is reversed phased HPLC equipped with diode array detection (DAD) and MS detection (LC-DAD-MS). The reproducibility and high sensitivity offer reliable analytical data and thus decrease the time of analysis.[43] As shown in Figure 3, the present reverse-phase isocratic HPLC method separated the three main carotenoids with fine resolution and in a short time (14 min). The retention times of lycopene and β-carotene in the sample were in accordance with genuine standards. The UV–VIS spectra of the carotenoids also serve as a helpful source for their identification and the DAD allows the UV–Vis spectrum of each component to be determined online. Both the wavelengths of maximum absorption (λmax) and the shape of the spectra are feature of each carotenoid. Derived from the LC-MS interpretation, bacterioruberin (the main carotenoid), lycopene and β-carotene were identified in the TBZ112 extract.

In comparison between this study with our previous report[6] mentioned in introduction section, *Halorubrum* sp. TBZ112 produced more total carotenoid than *Halorubrum* sp. TBZ126 in the same condition regarding culture medium, pH, temperature and salinity. In addition according to 16s rRNA gene sequencing and phylogenetic investigation, *Halorubrum* sp. TBZ112 despite of *Halorubrum* sp. TBZ126 does not 100% similarity with *Halorubrum chaviator* Halo-G^{*T} and based our observations, it can be introduced as new species or sub-species in *Halorubrum* genus by performing more and complete experiments. For example, optimum temperature for growth of *Halorubrum* sp. TBZ112 was recorded 31 °C but it was reported 37 °C for *Halorubrum chaviator* Halo-G^{*T}.[44]

Conclusion

The strain TBZ112 produced bacterioruberin as the dominant carotenoid and it can be proposed as a promising source for the mass production of bacterioruberin. Further studies are underway to reveal the optimal conditions for the enhanced production of carotenoids.

Acknowledgments

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Conflict of interests

The authors claim that there is no conflict of interest.

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