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REVIEW ARTICLE

Carotenoid Production by Halophilic Archaea Under Different Culture Conditions

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Abstract Carotenoids are pigments that may be used as colorants and antioxidants in food, pharmaceutical, and cosmetic industries. Since they also benefit human health, great efforts have been undertaken to search for natural sources of carotenoids, including microbial ones. The optimization of culture conditions to increase carotenoid yield is one of the strategies used to minimize the high cost of carotenoid production by microorganisms. Halophilic archaea are capable of producing carotenoids according to culture conditions. Their main carotenoid is bacterioruberin with 50 carbon atoms. In fact, the carotenoid has important biological functions since it acts as cell membrane reinforcement and it protects the microorganism against DNA damaging agents. Moreover, carotenoid extracts from halophilic archaea have shown high antioxidant capacity. Therefore, current review summarizes the effect of different culture conditions such as salt and carbon source concentrations in the medium, light incidence, and oxygen tension on carotenoid production by halophilic archaea and the strategies such as optimization methodology and two-stage cultivation already used to increase the carotenoid yield of these microorganisms.

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Introduction

Carotenoids have received great attention due to their different functions. They are the precursors of vitamin A in animals and humans. Carotenoids are employed in animal feed and as nutrient supplements, food colorants, and fragrances in the industry [52].

More than 750 carotenoids were isolated from natural sources and some of them, such as astaxanthin, may be obtained by chemical synthesis. However, the processes employed to produce carotenoids used in food have been strictly regulated since some of their by-products may present undesirable effects when consumed. Therefore, great efforts have been undertaken to search for natural sources of carotenoids, including microbial sources [12, 39, 44].

Several organisms, such as algae, yeasts and bacteria, are capable of producing carotenoids. In spite of the variety of natural and synthetic sources of carotenoids, only few, such as β-carotene and astaxanthin, are produced commercially by microbial cultivation [49]. The commercial utilization of microorganisms with biotechnological potential to produce carotenoids is restricted due to high production costs. However, costs may be minimized by

increase in carotenoid yield produced by the microorganisms, mainly through the optimization of production conditions, or by the use of inexpensive industrial by-products as nutrient sources [42, 44].

Halophilic archaea produce carotenoids according to culture conditions. The disruption of their cells to obtain the carotenoids may be simpler and faster since halophilic archaea cells lyse spontaneously in the presence of water and prevent any mechanical disintegration prior to extraction with organic solvents [3]. Therefore, current review summarizes the effect of different culture conditions such as salt and carbon source concentrations in the medium, light incidence, and oxygen tension on carotenoid production by halophilic archaea and the strategies such as optimization methodology and two-stage cultivation already used to increase the carotenoid yield by these microorganisms.

Halophilic Archaea

Halophilic archaea belong to the class *Halobacteria* which contains the order *Halobacteriales* and the family *Halobacteriacea*e. There is a proposal for the inclusion of two more orders, *Natrialbales* and *Haloferacales*, each one containing a single family, *Natrialbaceae* and *Haloferacaceae*, respectively, within the class *Halobacteria* [22]. Halophilic archaea are aerobic heterotrophic organisms. Most species require 1.7 M (9.9 %, w/v) to 2.5 M (14.6 %, w/v) NaCl for growth and structural stability, while some species are capable of inhabiting regions with salinity as high as 5.5 M (32.1 %, w/v) NaCl [40, 48].

The archaea cell wall presents a variety of chemical compounds such as polysaccharides, proteins, and glycoproteins. In the halophilic archaea cell wall, the carboxyl groups from glycoproteins with high contents of negatively charged acidic amino acids (aspartate and glutamate) as well as sulfate groups bind to sodium ions that are in high concentration in the environment and help the cell wall's stabilization. When sodium ions are diluted, the negatively charged regions of the halophilic archaea surface tend to repel themselves and cause cellular lysis [40, 41].

Halophilic archaea mainly accumulate KCl in an equivalent concentration to that of NaCl in the extracellular environment to maintain the positive aqueous equilibrium [48, 51]. Some haloalkaliphilic archaea, which grow under high salt and high pH conditions, may also accumulate the disaccharide sulfotrehalose [15].

Halophilic archaea may be a source of enzymes, such as amylases, amyloglucosidases, proteases, and lipases, that may be used in the processes that require macromolecules degradation in high salt concentration. They may be utilized in biopolymer production such as polysaccharides and polyesters. Further, the polysaccharides may be employed to modify rheological properties of aqueous systems and to stabilize the viscosity of solutions acting as thickener, gelling, and emulsifying agents. The polyesters may be used in the biodegradable production of plastics. Some halophilic archaea may also be a source of bacteriorhodopsin, an integral membrane protein that may be employed in the production of computer memories and in the synthesis of voltaic cells (artificial membranes capable of picking up the sunlight, using it in electricity production). They may produce lipids to be used as food additives or liposomes for drug, genes, and agents for cancer visualization delivery systems. The halophilic archaea are also capable of producing isoprenoid compounds such as carotenoid pigments [13, 46, 59].

Carotenoids Produced by Halophilic Archaea

Carotenoids are liposoluble pigments responsible for the yellow-orange or orange-red colors of plants, algae, microorganisms, and animals. Animals cannot produce carotenoids but they obtain them from the diet [9].

The carotenoids bacterioruberin, carotene, lycopene, canthaxanthin, 3-hydroxy-echinenone, lycopersene, phytoene, phytofluene, and 2-isopentenyl-3,4-dehydrorhodopin have already been identified in halophilic archaea [1, 2, 17, 19, 29–31, 33, 34, 37, 54, 56, 62, 65]. Some of these carotenoids may be found at low concentrations, suggesting they may be precursors from other carotenoids [47].

Usually, bacterioruberin is the main carotenoid from halophilic archaea [1, 19, 28, 29, 33, 55]. The carotenoid and its derivatives—monoanhydrobacterioruberin, bisanhydrobacterioruberin, and trisanhydrobacterioruberin—present 50 carbon atoms and may be found as *trans* and *cis* isomers (5-*cis*-bacterioruberin, 9-*cis*-bacterioruberin, 13-*cis*-bacterioruberin, 15-*cis*-bacterioruberin, and 5-*cis*,9′-*cis*-bacterioruberin) [9, 43, 55].

Halophilic archaea probably synthesize carotenoids through the mevalonate pathway. Units containing 5 carbon atoms (isopentenyl diphosphate and its isomer dimethylallyl diphosphate, both derived from acetyl-CoA) undergo polycondensation to form the prenyl chains of carotenoids, such as lycopene (Online Resource 1), considered the carotenoid prototype. The steps of this pathway and the enzymes responsible for these steps are still under study [18, 24, 45]. Bacterioruberin (Online Resource 2) synthesis may occur by the addition of prenyl groups in each end of the lycopene chain with the consequent addition of the hydroxyl groups [50]. However, in *Haloarcula japonica* JCM 7785^T, the addition of a prenyl group and a hydroxyl group in the lycopene chain may occur concomitantly and may be catalyzed by the same enzyme.

Afterward, this enzyme catalyzes the addition of the second prenyl group and another hydroxyl group to the molecule. The other two hydroxyl groups may be added only after the end of the lycopene molecule elongation [67]. Therefore, bacterioruberin synthesis may be similar but different according to the archaea strain.

Besides archaea, bacterioruberin has been found in the bacteria *Rubrobacter radiotolerans* [57], *Arthrobacter agilis* [20], and *Kocuria rosea* [11].

Bacterioruberin presents important biological functions in halophilic archaea. It acts as cellular membrane reinforcement since it increases membrane rigidity and decreases water permeability [19, 35]. It also protects the microorganism from DNA damaging agents such as ionizing radiation, ultraviolet radiation, and hydrogen peroxide [47, 60], probably due to its antioxidant capacity. Mandelli et al. [43] showed that extracts of *Halococcus morrhuae* and *Halobacterium salinarum* cells with bacterioruberin and its derivatives have high antioxidant capacity.

Studies on the application of carotenoids produced by halophilic archaea are scarce. It is highly relevant to study halophilic archaea since these microorganisms have shown a high biotechnological potential and their carotenoids may be an alternative source of pigments.

Effect of Culture Conditions on Carotenoid Production by Halophilic Archaea

Most literature on halophilic archaea-derived carotenoids deals with research and elucidation of lipid composition of the microorganisms, with few research work on the cultivation of these archaea for carotenoid production.

Nutritional and physical factors may influence the carotenoid synthesis by halophilic archaea. Table 1 describes the archaea that have already been cultivated to carotenoids production; the conditions in which they were cultivated and the analytical techniques used to identify and/or quantify the carotenoids produced.

Effect of Salts

Salt concentration in culture medium of halophilic archaea was evaluated by several researches (Table 1). Strain R-4, currently called *Haloferax mediterranei* ATCC 33500, produced about 20 times more pigments in liquid medium containing 15 % total salts than in medium containing 25 % total salts (based on the same amount of cell protein) [53]. The same strain was studied by Kushwaha et al. [31]. Cells grown in a medium with 15 % total salts produced 420 μg bacterioruberins g^{-1} cell protein, whereas those grown in a medium with 20, 25, and 35 % total salts produced 80, 22, and 30 μg bacterioruberins g^{-1} cell protein, respectively. Moreover, despite the highest amounts of monoanhydrobacterioruberin, bisanhydrobacterioruberin, and β-carotene had also been produced in the medium containing 15 % total salts, their concentrations were not higher than 65 μg g^{-1} cell protein. In contrast, when the same authors used archaea *Halobacterium cutirubrum*, currently called *Halobacterium salinarum* ATCC 33170, the cells did not form any C_{40} or C_{50} carotenoid pigments in the same medium with 15 % total salts. However, in a medium with 20, 25, and 35 % total salts, the cells produced more than 1400 μg bacterioruberins g^{-1} cell protein.

D'Souza et al. [14] also studied the strain *Hfx. mediterranei* ATCC 33500 in media with 20, 15, 10, 5, and 0 % NaCl and demonstrated that decrease in NaCl concentration followed increase in pigment content, whereas extracts of cells grown at 20, 10, and 5 % NaCl showed the pigment ratio of 1:5:9, respectively. The red pigment was identified as bacterioruberin. It has been suggested that *Hfx. mediterranei* could produce bacterioruberin to stabilize cell membrane and reduce cell lysis as a response to the stress caused by low salt concentration (lower than 20 % NaCl).

The cultivation of *Haloferax volcanii* strain WFD11 (DSM 5716) under low (12.5 %, w/v) and high (20.6 %, w/v) NaCl concentrations revealed that archaea produced 1.6- to 1.7-fold more carotenoids, per cell, in media with low salt content than with high salt content [6].

When *Halobacterium* strain SP-2 and *Halorubrum* strain SP-4 were cultivated at a salinity range between 150 and 300, they exhibited increase in pigmentation as salinity decreased. The two strains produced the highest pigment concentration at 150 salinity and the predominant pigment was attributed to the C_{50} carotenoid bacterioruberin [63].

Contrastingly, *Haloferax alexandrinus* strain TM^T (JCM 10717^T) cultivated in media between 0 and 35 % (w/v) NaCl showed higher carotenoid production in a medium with 25 % NaCl. Growth and carotenoid production did not occur at less than 10 % NaCl [2]. Using the same archaea and the same medium added with trace elements, Asker et al. [1] verified that the main C_{40} carotenoids produced were β-carotene, 3-hydroxy-echinenone, and canthaxanthin, and the C_{50} carotenoids identified were trisanhydrobacterioruberin, monoanhydrobacterioruberin, bacterioruberin isomers, and bacterioruberin. Bacterioruberin proportions at 15 % (w/v) NaCl were 60.28 % bacterioruberins, 3.55 % bacterioruberin isomers, 4.37 % monoanhydobacterioruberin, and 3.83 % trisanhydrobacterioruberin of total carotenoids in the culture medium which became deep red. In 20 and 25 % (w/v) NaCl media, the productivity of bacterioruberins decreased 8 % when compared to medium with 15 % NaCl. Furthermore, in a

Table 1 Culture conditions of halophilic archaea and analytical techniques for their carotenoid identification and/or quantification

Halophilic archaea	Media and culture conditions	Culture conditions tested	Techniques for carotenoid analysis
Strain R-4 (*Haloferax mediterranei* ATCC 33500)	Composition of a medium with 20 % salt content (%, w/v): 15.6 % NaCl, 1.3 % $MgCl_2{\cdot}6H_2O$, 2 % $MgSO_4{\cdot}7H_2O$, 0.1 % $CaCl_2{\cdot}6H_2O$, 0.4 % KCl, 0.02 % $NaHCO_3$, 0.05 % NaBr, 1 % yeast extract; 100 mL medium in 500 mL Erlenmeyer flasks, 38 °C, magnetic shaking [53]	15, 20, and 25 % salts	Spectrophotometry
Strain R-4 (*Haloferax mediterranei* ATCC 33500) *Halobacterium cutirubrum* (*Halobacterium salinarum* ATCC 33170)	Medium composed (%, w/v) of inorganic salts 19.4 % NaCl, 1.6 % $MgCl_2$, 2.4 % $MgSO_4$, 0.1 % $CaCl_2$, 0.5 % KCl, 0.02 % $NaHCO_3$, 0.05 % NaBr and 0.5 % yeast extract; pH 7.3, 38 °C, 4 days [31]	15, 20, 25, and 35 % total salt	Thin-layer chromatography Spectrophotometry
Haloferax mediterranei ATCC 33500	Synthetic salts medium containing 20 % NaCl, 0.5 % glucose and 0.05 M Tris–HCl; pH 7.2, 72 h [14]	0, 5, 10, 15, and 20 % NaCl	Thin-layer chromatography with densitometry Spectrophotometry
Haloferax volcanii strain WFD11 (DSM 5716)	Medium containing (%, w/v) 12.5 % (2.1 M) or 20.6 % (3.5 M) NaCl, 4.5 % $MgCl_2{\cdot}6H_2O$, 1 % $MgSO_4{\cdot}7H_2O$, 1 % KCl, 0.134 mL 10 % $CaCl_2{\cdot}2H_2O$, 0.3 % yeast extract and 0.5 % tryptone; 42 °C, vigorous shaking [6]	12.5 and 20.6 % NaCl	Spectrophotometry
Halobacterium strain SP-2 *Halorubrum* strain SP-4	Modified complete medium (CM) containing (%, w/v) 1 % yeast extract, 0.75 % acid-hydrolyzed casein and brine water (salinity 250); pH 7.2, 100 mL medium in 250 mL Erlenmeyer flasks, 37 °C, 150 rpm, 11 days [63]	Salinities of 150, 200, 250, and 300 pH 6, 7, 8 and 9	Spectrophotometry Thin-layer chromatography High-performance liquid chromatography with ultraviolet–visible detector
Strain TM (*Haloferax alexandrinus* JCM 10717)	Complex medium containing (%, w/v) 1 % yeast extract, 0.75 % casamino acids, 25 % NaCl, 4 % $MgSO_4{\cdot}7H_2O$, 0.2 % KCl and 0.3 % trisodium citrate; pH 7.2, 100 mL medium in 500 mL Erlenmeyer flasks, 37 °C, 240 rpm, 7 days [2]	0, 5, 10, 15, 20, 25, and 35 % NaCl 0, 1, 2, 3, 4, 5 and 6 % $MgSO_4{\cdot}7H_2O$, $MgCl_2$ and Na_2SO_4 0, 0.1, 0.2, 0.3, 0.4, 0.5, and 1 % KCl Absence and presence of trace elements (0.23 mg $FeCl_2$, 0.7 mg $CaCl_2{\cdot}7H_2O$, 0.03 mg $MnSO_2{\cdot}H_2O$, 0.044 mg $ZnSO_4$, 5 μg $CuSO_4{\cdot}5H_2O$ per 100 mL) Cultivation time (1 to 8 days) Temperature 15 °C to 55 °C (in 5 °C increments) pH 4.5 to 9.0 Absence and presence of stirring Medium volumes (100 mL, 200 mL, 300 mL, and 400 mL in 500 mL Erlenmeyer flasks) Light illumination	High-performance liquid chromatography with ultraviolet–visible detector Spectrophotometry

Table 1 continued

Halophilic archaea	Media and culture conditions	Culture conditions tested	Techniques for carotenoid analysis
Haloferax alexandrinus strain TM (JCM 10717)	Standard growth medium containing (%, w/v) 1 % yeast extract, 0.75 % casamino acids, 25 % NaCl, 4 % $MgSO_4 \cdot 7H_2O$, 0.2 % KCl, 0.3 % trisodium citrate and 1 mL of a trace element solution (2.3 mg $FeCl_2 \cdot 4H_2O$, 7 mg $CaCl_2 \cdot 7H_2O$, 0.3 mg $MnSO_2 \cdot H_2O$, 0.44 mg $ZnSO_4$, 0.05 mg $CuSO_4 \cdot 5H_2O$ per 100 mL); pH 7.2, 37 °C, 6-L cultures, 6 days [1]	10, 15, 20, and 25 % NaCl	Thin-layer chromatography Spectrophotometry High-performance liquid chromatography with ultraviolet–visible detector Electron ionization-mass spectrometry
Halobacterium cutirubrum NRC 34001 (*Halobacterium salinarum* ATCC 33170) *Halobacterium halobium* NRC 34020 (*Halobacterium salinarum* ATCC 43214)	BSMK medium: 15 amino acids, nucleotides, and the salts (%, w/v) 25 % NaCl, 0.2 % KCl, 0.5 % NH_4Cl, 2 % $MgSO_4 \cdot H_2O$, 0.01 % KNO_3, 0.005 % K_2HPO_4, 0.005 % KH_2PO_4, 0.05 % sodium citrate, 3×10^{-5} % $MnSO_4 \cdot 7H_2O$, 7×10^{-4} % $CaCl_2 \cdot 7H_2O$, 4×10^{-6} % $ZnSO_4 \cdot 7H_2O$, 2.3×10^{-4} % $FeCl_2$, 5×10^{-6} % $CuSO_4$; pH 6.5, usually 100 mL medium in 500 mL Erlenmeyer flasks, 37 °C, 7 days, with shaking	0, 0.1, 0.5, 1, and 2 % glycerol 0, 0.1, 0.5, 2, and 4 % glucose Absence and presence of light	Spectrophotometry Thin-layer chromatography
	Complex medium: casamino acids, yeast extract, the salts (%, w/v) 25 % NaCl, 0.2 % KCl, 2 % $MgSO_4 \cdot 7H_2O$ and other salts; usually 100 mL medium in 500 mL Erlenmeyer flasks, 37 °C, 7 days, with shaking [21]	0.1 % glycerol 2 % glucose	
Halobacterium salinarum JCM 10927	25 % NaCl, 0.15 % casamino acids, 0.03 % trisodium citrate, 0.025 % glutamic acid, 2 % $MgSO_4 \cdot 7H_2O$ and 0.2 % KCl; pH 7.0, 250 mL medium in 500 mL Erlenmeyer flasks, 37 °C, gentle stirring, until early stationary phase [16]	Absence and presence of light (60 μmol photons.m^{-2}.s^{-1} from fluorescent lamps) Low (N_2 bubbling) and high oxygen tension	High-performance liquid chromatography with photodiode-array detector
Halorubrum sp. TBZ126	Marine broth containing (%, w/v) 0.59 % $MgCl_2 \cdot 7H_2O$, 0.324 % $MgSO_4$, 0.18 % $CaCl_2$, 0.055 % KCl, 0.016 % $NaHCO_3$, 0.008 % KBr, 0.0034 % $SrCl_2$, 0.0022 % H_3BO_3, 0.0004 % Na_2O_3Si, 0.00024 % NaF, 0.00016 % NH_4NO_3, 0.0008 % Na_2HPO_4, 0.5 % peptone, 0.1 % yeast extract and various NaCl concentrations; 90 mL medium in 250 mL Erlenmeyer flasks, 120 rpm, 9 days [23]	Temperature 15 °C, 22 °C, 32.5 °C, 43 °C, and 50 °C pH 4, 5.2, 7, 8.7, and 10 2.5, 8.8, 16.25, 24 and 30 % NaCl Dark and under constant white fluorescent light (36-Watt Dulux® fluorescent lamp)	Spectrophotometry High-performance liquid chromatography with diode array detector Mass spectrometry
Haloferax mediterranei ATCC 33500	First stage: (%, w/v) 1 % glucose, 15.6 % NaCl, 1.3 % $MgCl_2 \cdot 6H_2O$, 2 % $MgSO_4 \cdot 7H_2O$, 0.1 % $CaCl_2 \cdot 6H_2O$, 0.4 % KCl, 0.02 % $NaHCO_3$, 0.05 % NaBr and 0.5 % yeast extract; pH 7, 15 L medium in 20-L fermenter jar, 37 °C, 150 rpm, 1 vvm[a] (15 nL min^{-1}) aeration Second stage: 20 % NaCl (w/v), 0.1 % glucose (w/v) and 50 mM Tris–HCl; pH 7.2, 500-mL Hinton's flasks, 37 °C, 120 rpm for various times [19]	1st stage: growth stages of 48 h, 60 h and 80 h 2nd stage: 5, 10 and 20 % NaCl 0, 2, 8, 10, 12, 16, and 20 % $MgSO_4 \cdot 7H_2O$ 0.1, 0.5, 1 and 2 % glucose and CH_3COONa Cultivation time (24 h, 36 h, and 48 h)	Thin-layer chromatography Spectrophotometry High-performance liquid chromatography with diode array detector

[a] Volume of air per volume of culture medium per minute

20 % NaCl medium, the β-carotene concentration increased to 10 % of total carotenoids, while in 25 % (w/v) NaCl media, the productivity of canthaxanthin increased between 30 and 40 % of total carotenoids. As D'Souza et al. [14] have already proposed, Asker et al. [1] also suggested that *Hfx. alexandrinus* strain TM^T tended to accumulate more bacterioruberins when cells were exposed to low NaCl concentration to prevent cell lysis.

Therefore, the kind of pigment produced by halophilic archaea and its concentration may be related to the salt requirement of the microorganism. The archaea *Haloferax mediterranei* ATCC 33500 and *Halobacterium salinarum* ATCC 33170, studied by Kushwaha et al. [31] and cultivated in the same medium and under the same conditions (Table 1), showed higher carotenoid production in different NaCl concentrations of 15 and 25 %, respectively.

Moreover, availability of certain nutrients may also affect carotenoid production by halophilic archaea, whereas *Halobacterium salinarum* ATCC 33170 and *Haloferax alexandrinus* strain TM^T (JCM 10717^T) produced more carotenoid pigments in the same 25 % NaCl concentration, albeit in media with different nutrients (Table 1).

Effect of Carbon Sources

Gochnauer et al. [21] investigated the influence of glucose on carotenoid production by *Hbt. cutirubrum* NRC 34001 and *Halobacterium halobium* NRC 34020, currently called *Hbt. salinarum* ATCC 33170 and *Hbt. salinarum* ATCC 43214, respectively. The highest bacterioruberin and β-carotene productions occurred in a medium with 0.5 % glucose among the glucose concentrations tested (0.1, 0.5, 2, and 4 %) in a chemically defined medium (BSMK) (Table 1). In a 4 % glucose medium, pigmentation was inhibited. Besides glucose, the effect of glycerol (0.1, 0.5, 1, and 2 %) on pigment formation was also studied (Table 1). Production of C_{50} red pigments decreased when glycerol was added, whereas C_{40} carotenes seemed to increase. When another medium, the complex medium, was employed (Table 1), a 2 % glucose addition increased pigmentation, while the addition of 0.1 % glycerol had no effect on pigmentation. The authors suggested that glucose may stimulate the conversion of mevalonate to C_{40} carotenes and bacterioruberins, while glycerol may inhibit the conversion of C_{40} carotenes to C_{50} bacterioruberins. Therefore, glucose may be an alternative to improve the carotenoid production by halophilic archaea. Kushwaha and Kates [32] investigated in greater detail the effect of glycerol on carotenoid production by *Hbt. cutirubrum*, currently called *Hbt. salinarum* ATCC 33170, and concluded that glycerol is an inhibitor of carotenogenesis in *Hbt. cutirubrum*.

Effect of Light and Oxygen Tension

Light is important for the regulation of carotenoid synthesis in a broad variety of organisms. Strains *Hbt. salinarum* ATCC 33170, *Hbt. salinarum* ATCC 43214 [21] and *Hfx. alexandrinus* TM (JCM 10717^T) [2] showed no difference in pigmentation when cultivated in the absence or in the presence of light (Table 1). However, *Hbt. salinarum* JCM 10927 cultivated in the dark and under light at 60 μmol photons m^{-2} s^{-1} (Table 1) evidenced that pigment composition alters according to light conditions. Bacterioruberin and β-carotene were identified in the dark-grown cells at concentrations 4.0 ± 0.4 nmol per 10^{10} cells and 2.7 ± 0.4 nmol per 10^{10} cells, respectively. In cells grown in the light, the content of bacterioruberin increased to 10.0 ± 0.2 nmol per 10^{10} cells, while β-carotene content decreased to 0.7 ± 0.0 nmol per 10^{10} cells. Actually, β-carotene biosynthesis was not influenced by light under the conditions tested since it is cleaved in its center to produce two retinal molecules, reducing the β-carotene concentration measured. Additionally, the authors evaluated simultaneously the influence of light and oxygen tension on carotenoid production by *Hbt. salinarum* JCM 10927 (Table 1). The bacterioruberin content in cells grown in the light and low oxygen tension (obtained after bubbling N_2 gas) decreased to 3.9 ± 0.4 nmol per 10^{10} cells. This result was expected since bacterioruberin biosynthesis from lycopene involves hydroxylation reactions which depend on oxygen atoms. In the dark-grown cells, total carotenoid content did not show significant changes with low oxygen tension. Taking the mevalonate pathway for the carotenoid production into consideration, the authors concluded that the carotenoid synthesis up to lycopene is not influenced by oxygen decrease in the dark. However, low oxygen tension suppresses bacterioruberin biosynthesis and consequently increases β-carotene and/or retinal concentrations. The highest total carotenoid concentration of 15.2 nmol per 10^{10} cells was achieved in the presence of light and high oxygen tension and was assigned to bacterioruberin. Results foregrounded the hypothesis that bacterioruberin has a role in photoprotection [16].

In several microorganisms, light and oxygen may act as a specific inducer of carotenogenesis. However, under illumination, partial or total requirement by oxygen may vary between species and the presence of oxygen may be more efficient than the presence of air [4, 64].

Optimization of Culture Conditions

Asker and Ohta [2] cultivated *Hfx. alexandrinus* strain TM (JCM 10717^T) in various conditions using the one-factor-at-a-time approach, i.e., the optimum value from one condition tested was used in the next condition until all of them were optimized. Once the optimum NaCl concentration of 25 % was established, *Hfx. alexandrinus* was cultivated in media containing the salts $MgSO_4 \cdot 7H_2O$ (0–6 %, w/v), $MgCl_2$, or Na_2SO_4 (Table 1) to determine whether the cells required sulfate anion or magnesium cation for growth and carotenoid production. Growth and carotenoid production by the archaea were not verified in

media with less than 1 % $MgSO_4$ and both were low in media with more than 4 % $MgSO_4$. Since the archaea grew and produced carotenoids in media containing 1–4 % $MgSO_4 \cdot 7H_2O$, and grew slowly and produced low carotenoid contents in media with even 3 % (w/v) $MgCl_2$ but failed to grow in all the tested Na_2SO_4 concentrations, the authors concluded that *Hfx. alexandrinus* required magnesium and sulfate ions for growth and carotenoid production. Possibly the high requirement for magnesium may be explained by its use for cell division. The demand for sulfate ions may be due to their presence in the cell wall of halophilic archaea.

The evaluation of salt influence on carotenoid production by *Hfx. alexandrinus* further required the testing of KCl concentrations 0–1 % (w/v) (Table 1). The halophilic archaea could grow with little pigmentation in a medium without KCl. The cell and carotenoid production improved with the increase of KCl concentration up to 0.2 %, when maximum growth and carotenoid production by *Hfx. alexandrinus* occurred. A slight decrease in growth and carotenoid production was reported above this concentration. The requirement for KCl by the strain was already expected since the salt is essential for the osmotic equilibrium of halophilic archaea.

Increase in cell and carotenoid content was also detected in the presence of trace elements ($FeCl_2$, 0.23 mg; $CaCl_2 \cdot 7H_2O$, 0.7 mg; $MnSO_2 \cdot H_2O$, 0.03 mg; $ZnSO_4$, 0.044 mg; $CuSO_4 \cdot 5H_2O$, 5 μg per 100 mL), probably because these salts meet with the straiń s iron, manganese, and zinc requirements [2].

Once the salt composition of the medium was determined, the parameterś temperature and pH were tested at a range between 15 and 55 °C and between 4.5 and 9, respectively (Table 1). Temperature and pH are environmental factors significantly affecting growth and product formation by microorganisms. Temperature may alter the concentration of enzymes involved in carotenoid production and their concentration may modify the amount of carotenoid produced by the microorganisms [5]. Further, pH may control the macromolecules stability of the microorganisms. Its effect on cell growth and product formation may vary with different microorganisms, medium composition, and operating conditions [25]. *Hfx. alexandrinus* grew and produced carotenoids when cultivated between 20 and 50 °C as well as in pH rates between 5 and 7.5. The maximum growth and carotenoid production occurred at 37 °C and pH 7.2. Cell membrane of halophilic archaea at moderate temperature may be less permeable to the diffusion of Na^+ ions when compared to elevated temperatures. Consequently, archaea may maintain more easily the millimolar concentration of Na^+ ions inside the cell. Moreover, at neutral or near-neutral pH, the halophilic archaea may maintain their pH gradient and an optimal proton motive force which would not occur in environments with elevated pH [7]. This effect of pH on growth and pigment production by halophilic archaea is corroborated by *Halobacterium* strain SP-2 and *Halorubrum* strain SP-4 which were cultivated under a pH range between 6 and 9 and showed the highest cell and pigment concentrations under pH 7 and 8, respectively [63].

Oxygen supply may also be tested for the improvement of carotenoid production by the archaea since the aeration condition necessary for the optimum production of cells may be different from that favoring the production of a metabolite [61]. In shake-flasks, the oxygen supply depends on liquid volume and agitation speed of flask. Frequently, the oxygen transfer rate increases with the rise in agitation speed and the reduction in liquid volume of flask [36]. Under shaking condition (Table 1), *Hfx. alexandrinus* exhibited high growth and carotenoid production and, without shaking, revealed little growth and no pigment production. Maximum growth and carotenoid production also occurred in cultivation under shaking in 500 mL Erlenmeyer flasks containing 100 mL medium when compared to flasks containing 200 mL, 300 mL, and 400 mL medium (Table 1). Since the strain is aerobe, agitation and lower volume of medium in the flask increased the amount of dissolved oxygen and favored growth and pigmentation. The halophilic archaea *Hfx. alexandrinus* produced 2.06 mg total carotenoids g^{-1} dry cell (2.94 % β-carotene, 33.88 % canthaxanthin, and 63.17 % bacterioruberins) under optimum conditions, i.e., cultivation in 500 mL Erlenmeyer flask containing 100 mL of medium composed of 1 % yeast extract, 0.75 % casamino acids, 25 % NaCl, 4 % $MgSO_4$, 0.2 % KCl, and trace elements, pH 7.2, at 37 °C, under shaking for 6 days [2].

Hamidi et al. [23] also optimized the conditions for carotenoid production by a halophilic archaea. However, the effect of temperature, pH, and NaCl concentration on carotenoid production by *Halorubrum* sp. TBZ126 was evaluated through the response surface methodology in which conditions are varied together, instead of one-factor-at-a-time approach, and better responses may be obtained due to the synergic effects of the conditions. The parameters' range tested were temperatures from 15 to 50 °C, pH values from 4 to 10 and salinities from 2.5 to 30 % (w/v) of NaCl. The optimum conditions for cell and carotenoid production were 31 and 32 °C, pH value 7.51 and 7.94, 18.33 and 20.55 % (w/v) of NaCl, respectively. Therefore, the highest carotenoid concentrations also occurred under moderate temperature and near-neutral pH as occurred with *Hfx. alexandrinus* strain TM (JCM 10717^T) [2]. The optimized conditions for cell and carotenoid concentrations provided 0.90 ± 0.01 g cells L^{-1} and 11.71 ± 0.01 mg carotenoids L^{-1}, respectively. Rates increased when cells were cultivated in the presence of light under optimized

conditions. The maximum carotenoid concentration of 16.35 ± 0.01 mg L^{-1} was achieved in optimized condition for carotenoid production under light exposure, which corroborated the hypothesis that carotenogenesis is a photoprotection mechanism. Bacterioruberin was the carotenoid with the greatest amount (more than 96 % of total carotenoids), while lycopene and β-carotene were present in minor amounts (<2 % each).

Two-Stage Cultivation for Carotenoid Production

Halophilic archaea may also be cultivated in two stages and was performed with *Hfx. mediterranei* ATCC 33500. In the first stage, the archaea was grown in the culture medium with 15.6 % (w/v) NaCl, and the cells obtained were separated and used in the second-stage cultivation for the production of carotenoids (Table 1). The C_{50} carotenoids bacterioruberin, monoanhydobacterioruberin, bisanhydrobacterioruberin, and the C_{45} carotenoid 2-isopentenyl-3,4-dehydrorhodopin were identified in the cell extracts obtained from the second-stage cultivation. At decreasing NaCl concentration, the production of pigments improved. *Hfx. mediterranei* showed higher yield of pigments (0.117 A_{494nm} mL^{-1} broth) in medium containing 5 % (w/v) NaCl, which was similar to medium with 10 % (w/v) NaCl, when compared to medium containing 20 % (w/v) NaCl (0.056 A_{494nm} mL^{-1} broth) and to single-stage cultivation alone (0.095 A_{494nm} mL^{-1} broth). Since partial cells lysed with low NaCl concentration (5 %), different $MgSO_4$ concentrations (0–20 %, w/v) were used in the second-stage cultivation to modify the medium osmotic pressure. The production of pigments was enhanced with increasing $MgSO_4$ amounts. The highest production occurred with 8 % of the salt (0.342 A_{494nm} mL^{-1} broth). It was the first report that $MgSO_4$ together with low NaCl concentration would reduce cell lysis and increase pigment production by *Hfx. mediterranei*. Since the possible pathway for carotenoid synthesis is from mevalonic acid, mainly derived from acetate, the authors used CH_3COONa (0.1–2 %, w/v) instead of glucose as a carbon source in the second-stage cultivation to promote directly the generation of mevalonic acid and consequently C_{50} carotenoids. The use of 0.1 % CH_3COONa as carbon source yielded 0.604 A_{494nm} mL^{-1} broth of pigments representing an increase of 92 % when compared to the use of 0.1 % (w/v) glucose (0.314 A_{494nm} mL^{-1}). When glucose and CH_3COONa concentrations exceeded 2 % (w/v) and 1 %, respectively, the pigment yield was lower. Finally, cells cultivated in 80 h in the first stage revealed a pigment yield which was slightly higher in the second-stage cultivation than cells cultivated in 48 h and 60 h in the first stage. However, efficiency of carotenoid production was the highest when cells cultivated for 48 h in the first stage were used as inoculum for the 48-h second-stage cultivation [19].

Identification and Quantification of Carotenoids Produced by Halophilic Archaea

Carotenoids produced by halophilic archaea were mainly identified by spectrophotometry (ultraviolet–visible absorbance) after separation or not by thin-layer chromatography or high-performance liquid chromatography (Table 1). In general, carotenoids present a three-fingered absorption spectrum and, although absorption maximum rates for several carotenoids are known, the technique is not sufficient to their identification [8, 58]. Separating carotenoids by high-performance liquid chromatography prior to their analysis with absorbance detectors, which is the most widely analytical method for this purpose, also present limitations. Ultraviolet–visible spectra must be compared to authentic standards commercially available to identify all carotenoids in the sample. Moreover, the solvent used in the analysis may alter the characteristic absorption spectra of carotenoids precluding the identification. Finally, since some structurally related carotenoid molecules co-elute, their similar ultraviolet–visible spectra cannot allow their distinction [8, 52].

Therefore, the qualitative analysis of microbial carotenoids requires a robust, highly selective and sensitive method [27]. According to Schiedt and Liaaen-Jensen [58], carotenoids must be identified by at least their absorption spectrum, their chromatographic properties in two different systems, such as thin-layer chromatography and high-performance liquid chromatography, and their mass spectrum. Further, the increasing interest in analyzing carotenoids directly in biological samples without laborious sample preparation has led to the use of analytical tools such as mass spectrometry, nuclear magnetic resonance spectroscopy, and Raman spectroscopy [52].

The coupling of high-performance liquid chromatography with mass spectrometry provides the identification of carotenoids based on their molecular mass and their fragmentation pattern with high sensitivity and selectivity that are crucial for the identification and quantification of carotenoids [52, 66]. If further information is necessary on the carotenoid structure, such as the assignment of *cis* and *trans* isomers, nuclear magnetic resonance could help and could be combined to high-performance liquid chromatography [38]. Finally, Raman spectroscopy, a nondestructive technique, may be employed for direct detection and quantification of carotenoids with minimal sample volume and pretreatment. Nevertheless, other techniques of separation and identification may be necessary to

complement this technique due to the substances chemical similarities [10, 26]. Since only certain studies used such analytical tools (Table 1), researches on the characterization of carotenoids from halophilic archaea using high-sensitive and selective techniques are needed to establish the carotenoid composition of the strains.

Concluding Remarks and Perspectives

Carotenoid production by halophilic archaea is salt dependent. The highest carotenoid production probably occurs in different NaCl concentrations among the species according to their NaCl requirement. Components such as CH_3COONa and glucose, which are precursors from the mevalonate pathway, ions, and environmental conditions, such as temperature, pH, dissolved oxygen, and the light incidence, may also be altered to enhance carotenoid production. Therefore, these strategies combined to optimization methodologies may be applied in the cultivation of halophilic archaea to obtain higher carotenoid yields.

Further studies on identification of carotenoids produced by halophilic archaea are required, whereas not all studies described in this review have identified the carotenoids by techniques as mass spectrometry, nuclear magnetic resonance, and/or Raman spectroscopy. Investigations on the biological functions and the applicability of carotenoids from halophilic archaea are also necessary.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that no conflict of interest exists.

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