Chapter 12

Carotenoids' Production from Halophilic Bacteria

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Abstract

Carotenoids have received considerable attention due to their interesting industrial applications and, more importantly, their potential beneficial effects on human health. Halophiles comprise a heterogeneous group of microorganisms that need salts for optimal growth. The pigments produced by these halophilic organisms comprise phytoene, β-carotene, lycopene, derivatives of bacterioruberin, and salinixanthin. Here, we describe the procedure to obtain salinixanthin from the extremely halophilic bacterium *Salinibacter ruber*. Additionally, we describe the expression of the β-carotene biosynthetic genes *crtE*, *crtY*, *crtI*, and *crtB* from *Pantoea agglomerans* in the moderately halophilic bacterium *Halomonas elongata* obtaining a strain able to produce practically pure β-carotene. Thus, the use of these halophilic microorganisms as a source of carotenoids constitutes an important commercial alternative in the production of carotenoids from biological sources.

Key words: Carotenoid, Salinixanthin, β-Carotene, *crt* genes, Halophiles, *Salinibacter ruber*, *Halomonas elongata*, Genetic engineering

1. Introduction

Hypersaline environments constitute typical examples of environments with extreme conditions, not only due to their high salinity but also because they may be exposed to high or low temperatures, low oxygen conditions, and in some cases high pH values. Hypersaline environments can be classified as athalassohaline and thalassohaline. Thalassohaline waters are originated by seawater condensation while the athalassohaline by evaporation of inland surface water (1). The predominant groups of microorganisms inhabiting these hypersaline environments are moderately halophilic and extremely halophilic microorganisms. The extreme halophiles are well represented among the *Halobacteriaceae*, although some extremely halophilic bacteria have been described (1). In the last years, an increased

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interest in the extreme halophiles has been detected, probably due to their potential biotechnological uses (2, 3).

Saltern ponds are frequently colored due to the presence of pigmented microorganisms, including *Dunaliella*, rich in β-carotene, Haloarchaea producing mainly bacterioruberin, and halophilic Bacteria, such as *Salinibacter ruber* that produces the carotenoid called salinixanthin.

The halophilic green algae accumulate high concentrations of carotenoids, such as β-carotene, astaxanthin, and canthaxanthin. Among them, the best studied is *Dunaliella salina*, a flagellate species that grows in saline media and tolerates high temperature (up to about 40°C). These extreme growth conditions facilitate the cultivation system, being an attractive candidate for commercial production of β-carotene (4, 5). The main carotenoid accumulated by *D. salina* is the β-carotene, which accounted for about 20% of the total carotenoids and it is accumulated in globules, located in the interthylacoid space within the chloroplast of the cell (6).

The family Halobacteriaceae contains exclusively halophiles that live in natural environments containing high salt concentrations (as high as 5 M NaCl) and grow optimally at 3.4–5.1 M NaCl. Most haloarchaea are brightly red-orange due to a large amount of a red membrane consisting of carotenoid pigments (7). Among the accumulated carotenoids, the most represented are C-50 carotenoids, mainly bacterioruberin and its derivatives (monoanhydrobacterioruberin and bisanhydrobacterioruberin) (8). Other carotenoids found in minor amounts in Haloarchaea are lycopersene, *cis*- and *trans*-phytofluene, and *cis*- and *trans*-phytoene (9). The function of these pigments seems to be related to a protective effect against light damage in the visible and ultraviolet range of the spectrum and a reinforcement of the cell membrane. It has been demonstrated that the red bacterioruberin-containing wild type of *Halobacterium salinarum* survives better than the colorless wild type in strong light (10). In the genus *Halobacterium*, it is described a variation of pigmentation according to the salt concentration of the medium (11).

S. ruber is a halophilic bacterium present in significant number in the communities identified in NaCl-saturated saltern crystallizer ponds, coexisting with members of the family *Halobacteriaceae*. This bacterium contains a high proportion of carotenoids in its membrane; however, the carotenoid identified (salinixanthin) is a C-40 acyl glycoside carotenoid, not related to the C-50 bacterioruberins identified in haloarchaea (12, 13).

Both haloarchaea and extremely halophilic bacteria grow under non-aseptic conditions due to the high concentrations of salts necessary for cultivation, which prevent contamination by other microorganisms. On the other hand, the process to obtain the carotenoids is simple, being extracted directly from the cells without any mechanical operation, because NaCl concentration below 15% induces cell lysis (14). Thus, the use of these halophilic

microorganisms as a source of carotenoids constitutes an important commercial alternative in the production of carotenoids from biological sources.

2. Materials

1. *S. ruber* M31^T (CECT 5946) (Spanish Type Culture Collection, Valencia, Spain).
2. *Halomonas elongata* ATCC 33173^T (American Type Culture Collection, Manassas, VA, USA).
3. *Escherichia coli* DHα (15).
4. pAC-BETA-04 (16, 17) (see Note 1).
5. pBBR1MCS-2 (18) (see Note 2).
6. pRK600 (19) (see Note 3).
7. Saline medium: 195 g/L NaCl, 25 g/L $MgSO_4 \cdot 7H_2O$, 16.3 g/L $MgCl_2 \cdot 6H_2O$, 1.25 $CaCl_2 \cdot 2H_2O$, 5.0 g/L KCl, 0.25 g/L $NaHCO_3$, 0.625 g/L NaBr, and 1.0 g/L yeast extract. Adjust to pH 7.0. Autoclave at 121°C for 15 min.
8. SW30 medium: 234 g/L NaCl, 39.0 g/L $MgCl_2 \cdot 6H_2O$, 61.0 g/L $MgSO_4 \cdot 7H_2O$, 6.0 g/L KCl, 0.7 g/L NaBr, 0.2 g/L $NaHCO_3$, 1.0 g/L $CaCl_2 \cdot 2H_2O$, and 0.5% (w/v) yeast extract. Autoclave at 121°C for 15 min.
9. Luria–Bertani (LB): Add 10 g of bacto-tryptone, 5 g of yeast extract, and 10 g of NaCl into 1 L of deionized water. Autoclave at 121°C for 15 min.
10. LB-agar: LB and 20 g/L agar. Autoclave at 121°C for 15 min.
11. Orbital shaker.
12. Centrifuge Sorvall Evolution RC Superspeed (Thermo Scientific, Waltham, MA, USA).
13. SpeedVac™ concentrator (Thermo Scientific, Waltham, MA, USA).
14. Lyophilizer.
15. Water bath.
16. Heater block.
17. Buffer A: Methanol/acetone (3:7.3).
18. Buffer B: Acetone/hexane (1:1).
19. Buffer C: Acetone/hexane (2:3).
20. Buffer D: Methanol/ethylacetate/petrol (25:25:50).
21. Buffer E: Acetonitrile/methanol/chloroform (47:47:6).

22. Buffer F: Phenol:chloroform (24:24).
23. Buffer G: Methanol/dichloromethane (50:50).
24. Antibiotics: Rifampicin, kanamycin, and cloramphenicol.
25. *Eco*RI and appropriate buffer.
26. T4 DNA Ligase and appropriate buffer.
27. Spectrophotometer.
28. TLC LuxPlate® Silicagel 60 F (Merck, Darmstadt, Germany).
29. High-Performance Liquid Chromatography (HPLC) equipment.
30. Column Hypersil ODS (5 μm, 100× 4.6 mm) (Thermo Scientific, Waltham, MA, USA).
31. Carotenoids standards (Sigma, St. Louis, MO, USA).
32. Membrane filters (0.45 μm) (Millipore, Bedford, MA, USA).

3. Methods

Here, we describe two methods: (1) a method to obtain salinixanthin from an extremely halophilic bacterium (see Subheadings 3.1–3.3) and (2) a method to produce β-carotene in a recombinant moderately halophilic bacterium (see Subheadings 3.4–3.9).

3.1. Production of Salinixanthin by the Extremely Halophilic Bacterium *S. ruber M31*^{T}

S. ruber M31^T contains a high proportion of carotenoids in its membrane, producing colonies of similar appearance to haloarchaea (20). It has been demonstrated that the carotenoids in haloarchaea contribute to survive better in strong light in comparison to colorless wild type. The principal (>96% of total) carotenoid found in *S. ruber* M31^T is salinixanthin, a C-40 acyl glycoside carotenoid chemically related to the carotenoids found in *Rhodothermus marinus* and different from C-50 carotenoids produced by haloarchaea (21).

1. To grow *S. ruber* M31^T, inoculate homogeneously the bacterium onto round Petri dishes containing saline medium (see Note 4) (21) and incubate at 37°C during 7 days to obtain isolated colonies.
2. Inoculate liquid saline medium (2-L Erlenmeyer flasks containing 1 L of saline medium) with a single colony and incubate at 37°C on a rotary shaker (200 rpm) for 1 day.
3. Harvest the cells in the late-exponential growth phase (see Note 5) by centrifugation (30 min, 5,500×*g*, 4°C).
4. Dry the pellet in a SpeedVac™ concentrator.
5. When necessary, preserve samples by lyophilization and/or store in the dark at −20°C.

3.2. Extraction of Salinixanthin

General precautions for work with carotenoids need to be taken (22).

1. Mix 9.4 g of lyophilized cells from *S. ruber* with 15 mL of water to facilitate cell lysis.
2. Extract the carotenoids with 150 mL of solution A.
3. Dry the solution at reduced pressure.
4. Precipitate white contaminants with cold acetone (see Note 6).
5. Repeat this step until all water-soluble pigments are into the acetone.
6. Elute the carotenoids with solution B (23).
7. Take the solution almost to dryness (reduced pressure, room temperature).
8. Achieve preparative TLC (see Note 7) in solution C eluted with methanol. Fat-soluble pigments remain in the hexane.

3.3. Analysis of Carotenoids

A conclusive identification is a prerequisite in obtaining reliable data (see Note 8).

1. Absorption spectrum UV/visible: The carotenoid extracts are analyzed by scanning the absorbance in the wavelength region of 400–600 nm using a spectrophotometer. The total carotenoid content in the hexane extract is estimated by measuring the absorbance at 490 nm. The results are given as OD per 100 mL culture (24).
2. TLC analysis: The extracts are purified by two TLC steps on silica gel involving first 35% acetone in petrol to remove the carotenes and then solution D (25).
3. HPLC analysis: The composition of carotenoids in the resulting extracts is analyzed with HPLC equipped with diode array detector with detection wavelengths set to 450 and 480 nm using a Hypersil ODS column (5 µm, 100× 4.6 mm) and solution E with a flow rate of 1 mL/min as the mobile phase (26, 27). The standards are commercial carotenoids in ethylacetate for chromatography.

3.4. Construction of pBBR1BETA, Expressing the Gene crtEYIB and the cDNA Encoding IPP Isomerase

Engineering halophilic bacteria to produce carotenoids is a subject of great scientific and commercial interest. The aim of this method is the expression of a biosynthetic pathway in the moderately halophilic bacterium *H. elongata* ATCC 33173^T to produce the high-value product β-carotene. A gene cluster, consisting of the β-carotene biosynthetic gene crt*EYIB* from *Pantotea agglomerans*, and the cDNA encoding IPP isomerase from *Haematococcus pluvialis* (16, 17) are expressed in *H. elongata* ATCC 33173^T to produce β-carotene.

1. Digest the vector pBBR1MCS-2 (5,144 bp; Km^R) (10 µg) with *Eco*RI to prepare for directional cloning.

2. Digest the plasmid pAC-BETA-04 (~12.6 kb) with *Eco*RI to obtain a 10.1-kb fragment containing all five carotenoids' biosynthetic genes.
3. Purify the digested DNA by extraction with solution F and standard ethanol precipitation.
4. Prepare the ligation mixture (10 μL total volume): pBBR1MCS-2 digested with *Eco*RI, 10.1-kb *Eco*RI-fragment from pAC-BETA-04, 10× ligation buffer, DNA-free water, and T4 DNA ligase (see Note 9).
5. Incubate the reaction mixture overnight at 16°C or for 4 h at 20°C (see Note 10).

3.5. Expression of the Plasmid pBBR1BETA in E. coli

1. Add the transforming DNA (10 μL ligation) in 50 μL of the *E. coli* competent cells. Swirl the tubes gently several times to mix their contents. Store the tubes on ice for 30 min (see Note 11).
2. Transfer the tubes to a rack placed in preheated 42°C circulating water. Store the tubes in the rack for exactly 45 s. Do not shake the tubes (see Note 12).
3. Rapidly transfer the tubes to an ice bath. Allow the cells to cool for 1–2 min.
4. Add 800 μL of LB medium to each tube.
5. Warm the cultures to 37°C in a water bath, and then transfer the tubes to a shaking incubator set at 37°C.
6. Incubate the cultures for 45 min to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid (see Note 13).
7. Transfer the appropriate volume (50, 100, 200, and 300 μL) of transformed *E. coli* competent cells onto agar LB medium containing the appropriate antibiotic.
8. Store the plates at room temperature until the liquid has been absorbed.
9. Invert the plates and incubate them at 37°C. Transformed colonies should appear in 12–16 h.
10. Check the transformants (see Note 14).
11. The resultant plasmid was designated pBBR1BETA (Fig. 1).

3.6. Expression of the β-Carotene Genes in H. elongata ATCC 33173^T

To express the β-carotene genes in *H. elongata* ATCC 33173^T, the plasmid pBBR1BETA was introduced into a spontaneous rifampicin-resistant (Rf^R) mutant of *H. elongata* ATCC 33173^T (see Note 15) by conjugal transfer using pRK600 (Cm^R) as a helper plasmid (28). Conjugal transfer of plasmid is performed by filter mating on solid media.

1. Incubate overnight the donor strain (*H. elongata*/pBBR1BETA), the recipient strain (*H. elongata* Rf^R), and the helper strain

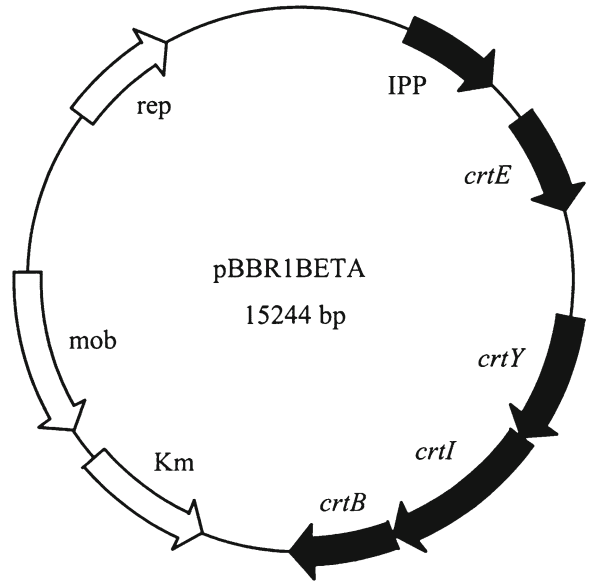

Fig. 1. Restriction map of the plasmid pBBR1BETA containing the β-carotene biosynthesis genes *crtE*, *crtY*, *crtI*, and *crtB* from *Pantoea agglomerans* and the gene encoding *Haematococcus pluvialis* IPP isomerase downstream of the strong bacterial *trc* promoter. The ORFs and the direction of transcription are shown by the large arrows. *Km* kanamycin resistance gene, *mob* mobilization gene, *rep* replication origin.

(*E. coli*/pRK600) in their respective medium and antibiotics: LB Km (50 μg/mL), SW2 Rf (25 μg/mL), and LB Cm (30 μg/mL), respectively.

2. Mix the culture of bacteria in proportion 1:1:1.
3. Centrifuge for 1 min at 13,000 × *g* to precipitate the cells.
4. Wash twice with 500 μL of SW2 medium to eliminate the rest of the antibiotics and resuspend in 150 μL of SW2.
5. The mating mixture is taking place on the surface of a sterile 0.45-μm pore-membrane filter onto a plate of SW2 medium.
6. Incubate at 37°C for 10–12 h.
7. Resuspend the cells of the filter in 1 mL of 20% (w/v) sterile glycerol and after appropriate dilutions, plate onto SW2 medium containing rifampicin (25 μg/mL), to counterselect the donor strain, and kanamycin (50 μg/mL), to select for *H. elongata* transconjugants carrying the plasmid pBBR1BETA (see Note 16).
8. Select one of the transconjugants of *H. elongata*. These colonies can be distinguished from the parental unconjugated strain by a subtle difference in the color. Colonies presumably containing β-carotene are deep orange-yellow in color.

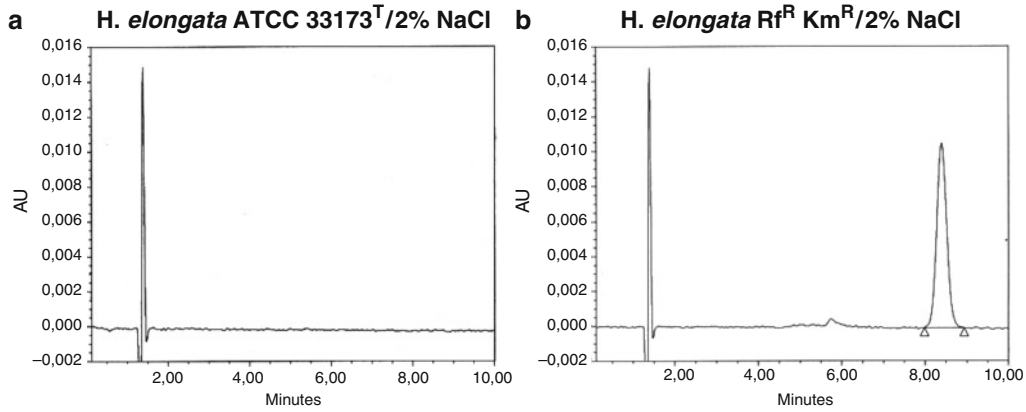

Fig. 2. HPLC profile of the carotenoids biosynthesized after flask fermentation by (**a**) *Halomonas elongata* TCC33173^T in SW with 2% (w/v) NaCl and (**b**) *H. elongata* Rf^R Km^R in SW with 2% (w/v) NaCl.

3.7. Culture of H. elongata for Optimal β-Carotene Production

Successful expression of the β-carotene biosynthetic pathway in *H. elongata* ATCC 33173^T opens the possibility of engineering halophilic bacteria for carotenoids' production. β-carotene is strongly dependent on NaCl concentration in the culture medium, with the highest production in the presence of 2% NaCl (Fig. 2).

1. Seed a Petri dish containing SW2 RfKm medium.
2. Inoculate 5 mL of SW2 RfKm medium with a single colony of the selected transconjugant *H. elongata* Rf^R Km^R.
3. Incubate the culture at 37°C.
4. Inoculate 50 mL of SW2 RfKm medium with 100 μL of *H. elongata* Rf^R Km^R.
5. Incubate overnight at 37°C under aerobic conditions with continuous shaking for 40 h.
6. Harvest the cells by centrifugation (10 min, 5,000 × *g*, 4°C).

3.8. Extraction of β-Carotene

General precautions for work with carotenoids need to be taken (22).

1. After harvesting the cells, wash the cell pellet with sterilized water, and extract it with 1 volume of solution G.
2. Dissolve the carotenoids in acetone and analyze by HPLC with a Hypersil ODS column (5 μm, 100× 4.6 mm) and buffer E as the mobile phase using a flow rate of 1 mL/min.

3.9. Analysis of β-Carotene

Perform the analysis of β-carotene following the method described in Subheading 3.3.

4. Notes

1. Plasmid pAC-BETA-04 (~12.6 kb) is constructed by the combination of plasmid pAC-BETA (10.6 kb) that includes the β-carotene biosynthetic genes *crtE*, *crtY*, *crtI*, and *crtB* (crt*EYIB)* from *P. agglomerans* (16) and a fragment ~2.0 kb containing the gene encoding *H. pluvialis* IPP isomerase downstream of the strong bacterial *trc* promoter (17).
2. Plasmid pBBR1MCS-2 (5,144 bp; Km^R) has several advantages over many of the currently available broad host range vectors: (1) it is relatively small (<5.3 kb); (2) it possesses an extended multiple cloning site (MCS); (3) it allows direct selection of recombinant plasmid molecules in *E. coli* via disruption of the LacZ alpha peptide; (4) it is mobilizable when the RK2 transfer functions are provided in *trans*; and (5) it is compatible with IncP, IncQ, and IncW group plasmids, as well as with ColE1- and P15a-based replicons (18).
3. pRK600 (Cm^R) is used as a helper plasmid (19).
4. To prepare the saline medium, use hot water and add the salt gradually. The last solution to be added must be $CaCl_2$ in order to avoid the precipitation of the solution.
5. To optimize the extraction of carotenoids, cells are grown until optical density 1.5 (late exponential phase).
6. As an alternative to precipitation in cold acetone, the white contaminants can be removed by this procedure: Add water to the raw extract dissolved in benzene to give two phases. Add methanol to precipitate the white contaminants in the hypophase. Remove the hypophase, repeat the procedure with increasing amounts of hexane in the epiphase, and stop before the carotenoid turned hypophasic.
7. Thin-Layer Chromatography (TLC) is a chromatographic technique used to separate mixtures. The process is similar to paper chromatography with the advantages of faster runs, better separations, and the choice between different stationary phases. Because of its simplicity and speed, TLC is often used for monitoring chemical reactions and for the qualitative analysis of reaction products.
8. In some studies using HPLC, the peaks of the chromatograms are designated as the signals for different carotenoids without concrete evidence of identification. This has led the world authorities establish minimum criteria for carotenoids' identification: (1) absorption spectrum UV/visible according to the suggested chromophore and (2) chromatographic properties consistent in two different systems: TLC and HPLC, including co-chromatography with authentic standard.

9. The molar ratio of plasmid vector to insert DNA fragment should be ~1:1 in the ligation reaction. The final DNA concentration should be ~10 ng/μL.
10. Depending on the commercial company could be differences, so the recommendation is to follow the indications of the manufacturer.
11. In the different experiments, it is essential to include positive controls to measure the efficiency of transformation, and negative controls to eliminate the possibility of contamination and to identify the potential causes of failure. Negative control: An aliquot of competent cells to which no DNA is added should be carried through the transformation experiment. The entire aliquot should be plated on a single agar plate containing the appropriate antibiotic used to select transformants. No bacterial colonies should grow on these plates or on a selective plate that received no bacteria. Positive control: An aliquot of competent cells should be transformed with a known amount of a standard preparation of circular superhelical plasmid DNA.
12. Heat shock is a crucial step. It is very important the cells to be raised to exactly the right temperature at the correct rate.
13. To maximize the efficiency of transformation, gently agitate (<225 cycles/min) the cells during the recovery period.
14. To check the transformant colonies, a commercial kit for plasmid DNA extraction can be used.
15. To obtain *H. elongata* spontaneous Rf^R mutants, *H. elongata* ATCC 33173^T has to be cultivated in SW2 rifampicin (25–100 μg/mL) plates and incubated at 37°C overnight. Colonies that appear in these plates correspond to *H. elongata* Rf^R mutants.
16. As a control for spontaneous mutations, plate both parental strains on the SW2 Rf Km medium.

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