

Selection and Taxonomic Identification of Carotenoid–Producing Marine Actinomycetes

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Abstract

Carotenoids are important pigments produced by plants and many microorganisms, including fungi, microalgae, cyanobacteria, and bacteria. Marine actinomycetes are a group of bacteria that produce a variety of metabolites with economic potential. Here, we describe a general method of selecting marine actinomycetes as carotenoids' producers. The screening is carried out at two levels: the first one involves a quick selection of strains by visual color inspection, and the second consists in the analysis of the extracts by HPLC. The taxonomic analysis of the producing strains gives us an overview of the groups of actinomycetes in which carotenoids can be found.

Key words: Carotenoids, Actinomycetes, Marine bacteria, Color, Taxonomic analysis

1. Introduction

Carotenoids are a family of yellow to orange-red terpenoid pigments synthesized by photosynthetic organisms and by some microorganisms, such as fungi, microalgae, cyanobacteria, and heterotrophic and prototrophic bacteria (1). These compounds are used as colorants, feed supplements, and nutraceuticals in the food, medical, and cosmetic industries (2). Carotenoids are naturally found in the chloroplasts and chromoplasts of plants and some other photosynthetic organisms, such as algae. Carotenoids are potent antioxidants that may have protective effects on the microorganisms against oxidative damage (3, 4). Animals do not produce carotenoids, so they have to get them through their diets.

Carotenoids conform a numerous group of compounds with over 600 known molecules. Their main function in phototrophic organisms is to absorb light energy for its use in photosynthesis, as well as to protect chlorophyll from photodamage. In humans, they act

as antioxidants, some of them as precursors in the biosynthesis of vitamin A (β -carotene, α -carotene, γ -carotene, and β -cryptoxanthin), and to protect the macula lutea of the eye. Carotenoids are used too as natural pigments in the aquaculture and poultry industry.

Actinomycetes in general and marine actinomycetes in particular are the most important producers of secondary metabolites as well as other products of industrial and pharmaceutical interest (5). While the terrestrial actinomycetes have been thoroughly searched since the discovery of the streptomycin by Waskman in 1943, the marine counterpart has focused little attention until recently. Nowadays, this group of organisms is being intensively studied, and new taxons have been isolated in sediments (6) or associated to invertebrates (7). Besides the production of secondary metabolites and enzymes, actinomycetes can produce non-soluble pigments that make them a target for the screening in search of new carotenoids or as overproducers of known ones.

The methods described here are of general applicability with any collection of marine bacteria. The growth media are suitable for the growth of the majority of marine actinomycetes. We describe first a quick identification of cultures by visual inspection with the potential for producing carotenoids. This procedure greatly diminishes the number of cultures that have to be screened by HPLC. A method for the identification of carotenoids is detailed. Due to the great number of different carotenoids, the complete identification of the compound will usually require methodologies as mass spectrometry and NMR, which are out of the scope of this chapter. Finally, a procedure for identifying the microorganisms through 16S rDNA sequence is given. This procedure works well with most marine actinomycetes.

2. Materials

2.1. Growth of Marine Actinomycetes

1. Artificial seawater salts (Hobby-Marin, Dohse Aquaristik AG, Gelsdorf, Germany).
2. 172b liquid medium: Add about 800 mL distilled water to a glass beaker. Weigh in 10 g of glucose, 20 g of soluble starch, 5 g of yeast extract, 5 g of tryptone, and 10 g of artificial seawater salts. Adjust to pH 7, and add 1 g of calcium carbonate (see Note 1). Make up to 1 L with distilled water. Autoclave at 121°C for 20 min.
3. 172b solid medium: 172b liquid medium and 15 g of agar. Autoclave at 121°C for 20 min.
4. ISP1 with marine salts: 5 g of tryptone, 3 g of yeast extract, and 10 g of artificial seawater salts. Make up to 1 L with distilled water. pH should be 7.0–7.2. Autoclave for 15 min at 121°C.

2.2. Liquid Fermentation and Extraction

1. Orbital shaker with temperature control. Additional humidity control is desirable.
2. Freezing and lyophilizing equipment.
3. Microcentrifuge.

2.3. Carotenoids' Analysis

1. Sample extraction cocktail: Dichloromethane: methanol: acetone (1:1:2; v/v).
2. Nylon filters 0.45 μm .
3. Mobile phase: Ethyl acetate:*n*-hexane (1:1; v/v).
4. Reverse-phase HPLC column: Nucleosil 5 μm NH_2 100A (250 \times 4.6 mm) (Phenomenex, Torrance, CA, USA).
5. HPLC equipment with PDA detector.

2.4. DNA Amplification

1. 27f primer: 5'-AGAGTTTGATCMTGGCTCAG-3', where M = C:A (1:1).
2. 1492r primer: 5'-TACGGYTACCTTGTTACGACTT-3', where Y = C:T (1:1).
3. DNeasy kit (Qiagen, Valencia, CA, USA).
4. Plastic microtubes (Eppendorf, Hamburg, Germany).
5. 10 \times Tuning buffer containing 25 mM Mg^{2+} .
6. dNTP mix: 0.5 mM each dATP, dTTP, dGTP, and dCTP.
7. Taq DNA polymerase.
8. Thermocycler.

2.5. Agarose Gel Electrophoresis

1. TBE: 10.8 g of Tris base, 5.5 g of boric acid, 20 mL of 0.5 M EDTA, and distilled water up to 1 L. pH 8.0 (see Note 2).
2. Ethidium bromide 10 mg/mL (see Note 3).
3. Agarose low electroendosmotic flow, electrophoresis grade (8).
4. Electrophoresis equipment.

2.6. 16S rDNA Sequencing

1. QIAquick gel extraction kit (Qiagen, Valencia, CA, USA).
2. DNA ladder 100 pb (BioLabs, Beverly, MA, USA).

3. Methods**3.1. Quick Identification of Producing Actinomycetes**

The actinomycetes can be screened in a fast way by the color of their colonies. The producing genera are spread in different suborders and classes of the order *Actinomycetales*. A non-exhaustive list of genera and colors (yellow, pink, orange, and red) that can be searched is shown in Table 1.

1. Grow the bacteria in solid medium 172b (see Note 4). Incubate the plates at 28°C for 7 days.

Table 1

List of genera and colors (yellow, pink, orange, and red) that can be found in carotenoid-producing marine actinomycetes

Basic color	Shade	Genus
Yellow	Pale-cream	<i>Actinoplanes</i> , <i>Agrococcus</i> , <i>Arsenicicoccus</i> , <i>Cellulomonas</i> , <i>Kocuria</i> , <i>Microbacterium</i> , <i>Rhodococcus</i> , <i>Serinicoccus</i> , <i>Streptomyces</i>
	Intense	<i>Rhodococcus</i>
	Yellow-orange	<i>Microcella</i> , <i>Rhodococcus</i>
	Yellow-greenish	<i>Kocuria</i> , <i>Leifsonia</i> , <i>Microbacterium</i> , <i>Rhodococcus</i>
Pink		<i>Arthrobacter</i> , <i>Gordonia</i>
Orange-red		<i>Blastococcus</i> , <i>Dietzia</i> , <i>Microcella</i> , <i>Micromonospora</i> , <i>Nocardia</i> , <i>Rhodococcus</i> , <i>Salinibacterium</i> , <i>Streptomyces</i> , <i>Verrucosispora</i>

2. Check the color of the colonies visually and make a photographic record (see Note 5).
3. Select the colonies that show the colors described in Table 1.
4. Preserve these cultures frozen to be used as inocula for liquid fermentation (see Note 6) (9).

3.2. Liquid Fermentation

Liquid fermentation for carotenoids' detection can be made in one step in flasks. 5 mL broths are enough for HPLC analysis. The broths are subjected to lyophilization for eliminating the water content. The desiccated broths are then extracted with a solvent mixture of adequate polarity in order to extract the carotenoids.

1. To prepare a fresh culture of the bacteria, inoculate the bacteria onto Petri dishes (9-cm diameter) containing 172b agar medium.
2. Incubate the Petri dishes at 28°C for 7 days.
3. Inoculate a 250-mL Erlenmeyer flask containing 30 mL of 172b liquid medium with the fresh culture (see Note 7). Incubate the flask at 28°C on a rotary shaker at 250 rpm for 96 h.
4. After 96 h of incubation, transfer a 5 mL sample of the fermentation broth to a 25-mL amber vial. Cap the vial with a lyophilization butyl rubber stopper and freeze it at -20°C. Store the vial at -20°C until needed for lyophilization (see Note 8).
5. Lyophilize the vial (see Note 9).

3.3. Carotenoids' Analysis

1. Add 15 mL of extraction cocktail to a lyophilized vial and shake for 20 min in an orbital shaker.
2. Filter through 0.45-μm nylon filters in HPLC vials.

3. Adjust sample temperature to 10°C and column temperature to 30°C.
4. Adjust mobile-phase flow to 1 mL/min in an isocratic elution mode.
5. Inject samples of 10 µL and run for 60 min.
6. Monitor the OD in the wavelength range from 200 to 700 nm.
7. Compare the HPLC profile obtained (RF and maximum of absorption) with the HPLC profile of the standards (Fig. 1). A typical HPLC profile of carotenoids is shown in Fig. 1.

3.4. DNA Amplification

The carotenoid-producing strains can be taxonomically identified by their 16S rDNA sequence. For that, the breakage of the cells is needed. The isolation of DNA from actinomycetes sometimes has problems since they are Gram-positive bacteria and possess a thick peptidoglycan layer in their cell walls. This thick layer can render some actinomycetes insensitive to lysozyme treatment. When this happens, several procedures can be used either for weakening the peptidoglycan layer so that it becomes sensitive to the lysozyme treatment or using another protocol for breaking the cells (10). Here, we describe a method for obtaining the DNA that works well with most actinomycetes. It usually produces DNA of enough quality to get good PCR amplifications of the 16S rDNA.

1. Grow the bacteria in ISP1 liquid medium with marine salts (50-mL-capacity flasks with 10 mL of medium) for 72 h at 28°C and 200 rpm.
2. Harvest the cells by centrifuging 1 mL of culture in a plastic microtube for 10 min at 5,000 × *g* (see Note 10).
3. Extract the DNA using the DNeasy kit.
4. Monitor the quality and amount of DNA by 0.8% half-strength agarose gel electrophoresis in TBE buffer and 5 µL of ethidium bromide (from a stock solution of 10 mg/mL) per 100 mL of gel solution.
5. Amplify by PCR the 16S rDNA genes using the eubacterial primers 27f and 1492r (11). Reaction mixture: full-strength tuning buffer, 200 nM primer 27f, 200 nM primer 1492r, 100 nM of each dNTP, 0.05 U of Taq polymerase, and 5 µL of DNA sample. PCR thermal cycle: 1 min at 94°C, and then 30 cycles of 10 s at 98°C, 30 s at 48°C, and 3 min at 72°C. Complete the reaction by incubating during 7.5 min at 72°C.

3.5. rDNA Sequencing

1. Purify the amplified fragments of DNA from agarose gels using the QIAquick gel extraction kit.
2. Analyze by electrophoresis in 0.8% agarose gel using a standard preparation (100 pb DNA ladder) to quantify the DNA by comparison. For that, a sample of the standard 100 pb DNA ladder is run in parallel to some dilutions of the DNA sample.

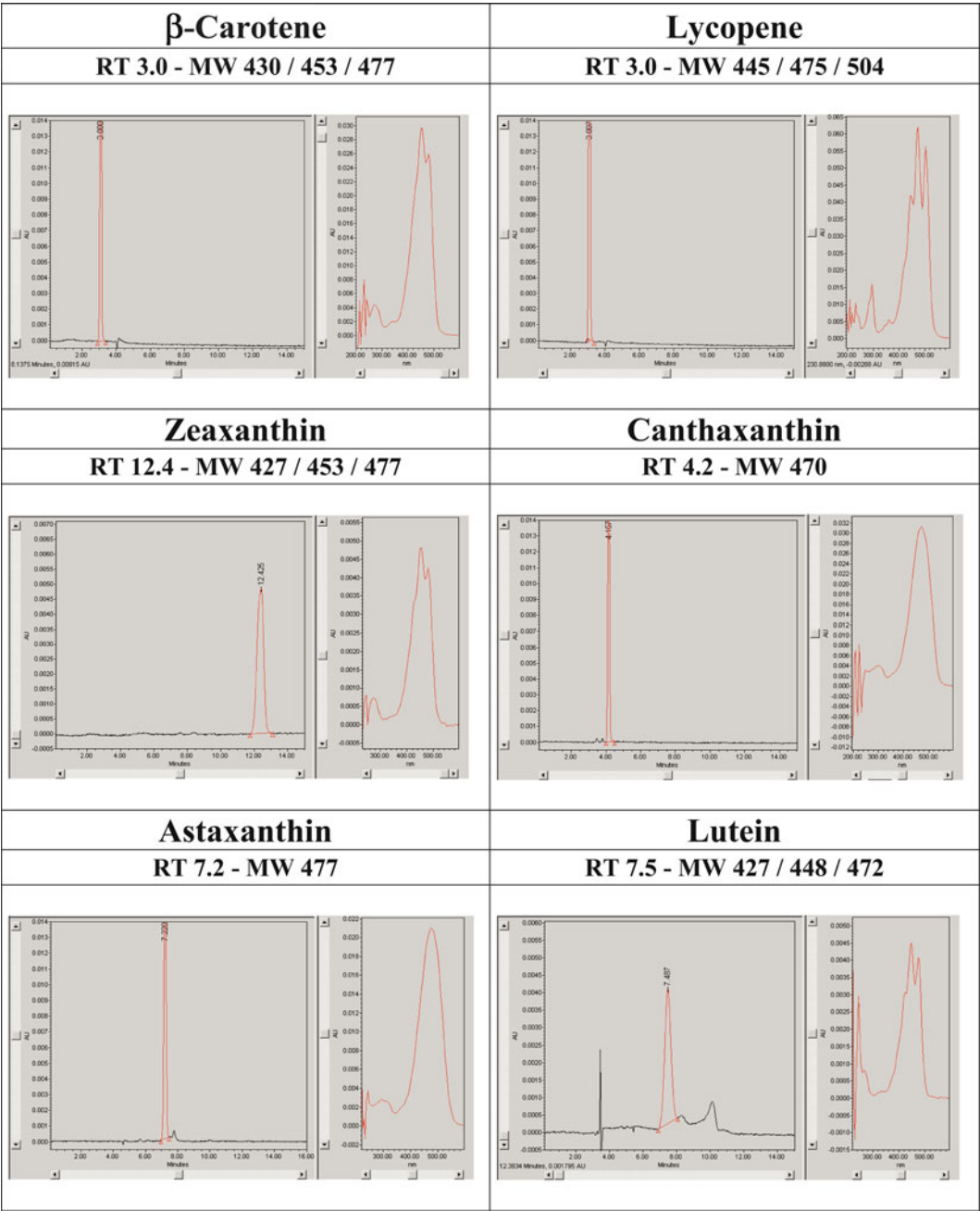


Fig. 1. Typical HPLC profile of some carotenoids produced by marine actinomycetes. *RT* retention time (min). *MW* maximum wavelength.

- The amount of the DNA is estimated for the intensity of the band compared to the standard.
3. Prepare the purified DNA fragments for sequencing according to the advice of the sequencing service (see Note 11).
 4. Analyze the sequences for the identification of the producing strains.

4. Notes

1. Calcium carbonate acts as a buffer counteracting the production of acids with carbonic anhydride. It must be always added after pH adjustment.
2. Prepare the solution five times more concentrated (5× TBE) and store it at room temperature to avoid precipitation.
3. Ethidium bromide is highly mutagenic. Wear double gloves. Decontaminate the solutions following Lunn and Sansone (12) or by incineration since ethidium bromide decomposes at 262°C.
4. 172b is a rich, non-sporulating medium. Aerial mycelium development masks the color of the colony, making more difficult to identify the cultures producing the carotenoids.
5. The photographs are very useful for comparing the colors of the colonies with the carotenoids' production and taxonomy of the cultures. In a continuous screen, it helps improving the selection of the colonies.
6. Actinobacteria, as most Gram-positive bacteria, survive well to freezing. For not very long periods of time, they can be frozen in the absence of protecting agents. Freezing the cultures on agar plugs is then recommended since the agar acts as a cryopreservative.
7. The aeration of the cultures when fermenting in 250-mL flasks with 30 mL of medium is good enough for most applications that demand high aeration rate. 30 mL of broth exceeds the amount needed simply for carotenoids' detection, but produces enough volume for other analysis. If only carotenoids' detection is needed, then 50-mL-capacity flasks containing 10 mL of medium would provide with a well-aerated culture.
8. Different lyophilizers will produce vacuum at different rates depending on the pump used and the volume of lyophilizing chamber. At the same time, the amount of filling of the vials and the amount of heat received by the vials to maintain the evaporation of the water are important factors in the lyophilization. Tests will have to be performed to ensure that the frozen broths do not melt during the process. Different volumes of broth could be tested.
9. The production of foaming substances during the fermentation can cause bubbling in some vials at the beginning of the lyophilization.
10. Young cultures of actinomycetes are more susceptible to lysozyme than older ones since the cells of the younger cultures

are actively reproducing and the level of cross-linkage in the peptidoglycan is smaller.

11. The sequencing service used in this work is Secugen S.L. (Madrid, Spain). It requests for 450 ng of amplified DNA and 1.5 µL of 5 µM primer 27f.

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