Chapter 8

Metabolic Engineering of *Mucor circinelloides* for Zeaxanthin Production

Marta Rodríguez-Sáiz, Juan-Luis de la Fuente, and José-Luis Barredo

Abstract

Mucor circinelloides is a β-carotene producing zygomycete amenable to metabolic engineering using molecular tools. The *crtS* gene of the heterobasidiomycetous yeast *Xanthophyllomyces dendrorhous* encodes the enzymatic activities β-carotene hydroxylase and ketolase, allowing this yeast to produce the xanthophyll called astaxanthin. Here we describe the fermentation of *X. dendrorhous* in astaxanthin producing conditions to purify mRNA for the cloning of the cDNA from the *crtS* gene by RT-PCR. Further construction of an expression plasmid and transformation of *M. circinelloides* protoplasts allow the heterologous expression of the *crtS* cDNA in *M. circinelloides* to obtain β-cryptoxanthin and zeaxanthin overproducing transformants. These two xanthophylls are hydroxylated compounds from β-carotene. These results show that the *crtS* gene is involved in the conversion of β-carotene into xanthophylls, being potentially useful to engineer carotenoid pathways.

Key words: Zeaxanthin, Astaxanthin, β-Carotene, β-Cryptoxanthin, Xanthophyll, Fermentation, *Xanthophyllomyces dendrorhous*, *Mucor circinelloides*

1. Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4-4′-dione; $C_{40}H_{52}O_4$) is a red xanthophyll (oxygenated carotenoid) with large importance in the aquaculture, pharmaceutical, and food industries. It is widely used as a feed additive to pigment flesh in salmon and trout aquaculture (1) and also conferring a characteristic coloration to crustaceans and some birds. It belongs to the carotenoids, a family of yellow to orange-red terpenoid pigments synthesized by photosynthetic organisms and by many bacteria and fungi (2) that are used as colorants, feed supplements, and nutraceuticals in the food, medical, and cosmetic industries (3).

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Despite the availability of a variety of natural and synthetic carotenoids, there is currently renewed interest in microbial sources (4). The green alga *Haematococcus pluvialis* and the heterobasidiomycetous yeast *Xanthophyllomyces dendrorhous*, the teleomorphic state of *Phaffia rhodozyma* (5–8), are currently known as the main microorganisms useful for astaxanthin production at the industrial scale. The improvement of astaxanthin titer by microbial fermentation is a requirement to be competitive with the synthetic manufacture by chemical procedures, which at present is the major source in the market.

The biosynthetic pathway of astaxanthin (Fig. 1) has been studied in *X. dendrorhous* (9–12). The *idi* gene encodes isopentenyl pyrophosphate (IPP) isomerase, which catalyzes the isomerization of IPP to dimethylallyl pyrophosphate (DMAPP) (13). The conversion of these isoprenoid precursors into β-carotene is catalyzed by four enzymatic activities: (i) geranylgeranyl pyrophosphate (GGPP) synthase (encoded by the *crtE* gene), which catalyzes the sequential addition of three IPP molecules to DMAPP to give the C20-precursor GGPP (14); (ii) phytoene synthase (encoded by the *crtYB* gene), which links two molecules of GGPP to form phytoene (15); (iii) phytoene desaturase (encoded by the *crtI* gene), which introduces four double bonds in the phytoene molecule to yield lycopene (16); and (iv) lycopene cyclase (also encoded by the *crtYB* gene), which sequentially converts the ψ acyclic ends of lycopene to β rings to form γ- and β-carotenes (15). Two additional enzymatic activities convert β-carotene into astaxanthin through several biosynthetic intermediates: a ketolase which incorporates two 4-keto groups in the molecule of β-carotene, and a hydroxylase which introduces two 3-hydroxy groups. In *X. dendrorhous*, both activities could be together as a single enzyme (astaxanthin synthetase; CrtS) encoded by the *crtS* gene, which sequentially catalyzes the 4-ketolation of β-carotene followed by the 3-hydroxylation (17). However, the expression of the *crtS* gene in a β-carotene producing strain of *Mucor circinelloides* revealed the lack of astaxanthin and ketolated intermediates (18, 19), which does not support that hypothesis. In contrast, two independent genes have been described in other astaxanthin producing microorganisms. Additionally, a cytochrome P450 reductase, encoded by the *crtR* gene, has been shown to have an auxiliary role to CrtS in *X. dendrorhous*, providing with the necessary electrons for substrate oxygenation (20). Expression of the *crtS* gene in a β-carotene producing strain of *Saccharomyces cerevisiae* did not lead to astaxanthin production, but coexpression of *crtS* and *crtR* genes resulted in the accumulation of a small amount of astaxanthin (21). The existence of a monocyclic pathway diverging from the dicyclic pathway at neurosporene and proceeding through β-zeacarotene, 3,4-didehydrolycopene, torulene, 3-hydroxy-3′,4′-didehydro-β, ψ-carotene-4-one (HDCO) to the end product 3,3′-dihydroxy-β, ψ-carotene-4,4′-dione (DCD) (Fig. 1) was also proposed (22).

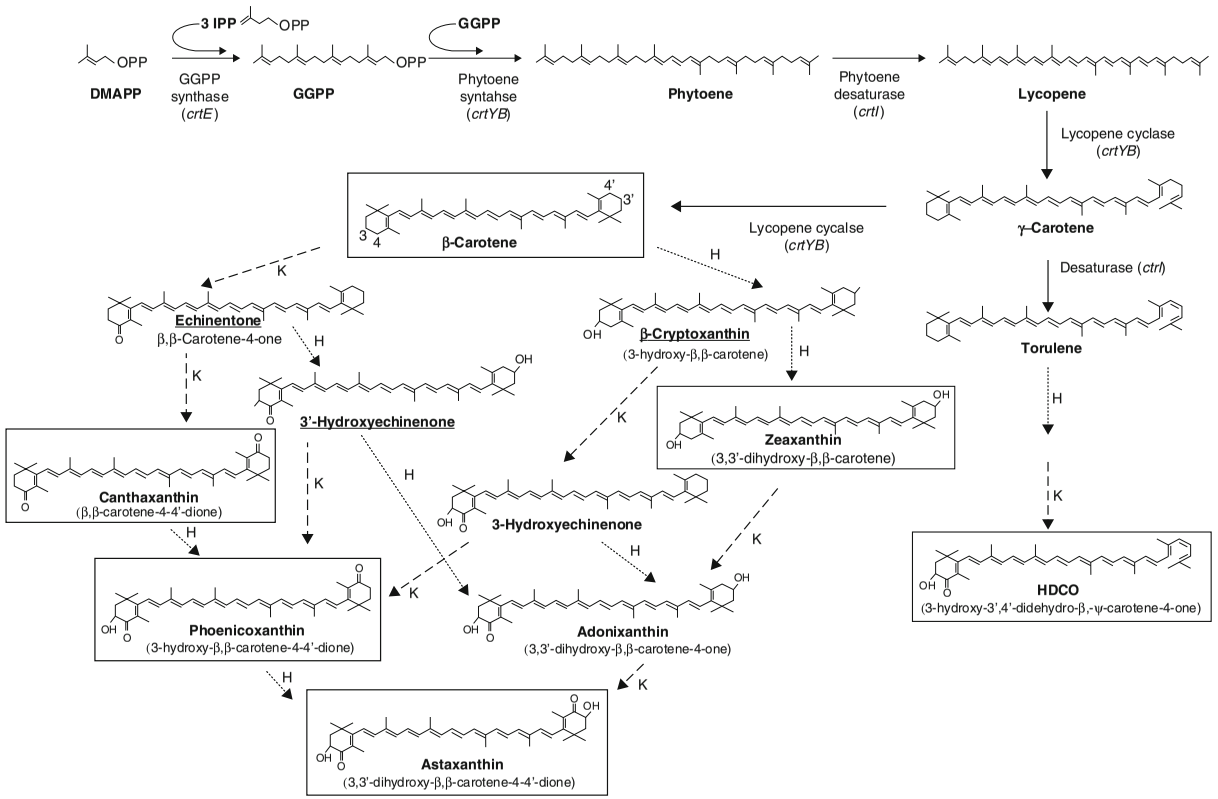

Fig. 1. Biosynthetic pathway leading to astaxanthin in *Xanthophyllomyces dendrorhous.* One molecule of dimethylallyl pyrophosphate (DMAPP) and three molecules of isopentenyl pyrophosphate (IPP) are coupled to form geranylgeranyl pyrophosphate (GGPP) by the GGPP synthase. Subsequently, two molecules of GGPP are joined by the phytoene synthase, encoded by the *crtYB* gene, to form phytoene. Afterwards, the phytoene desaturase, encoded by the *crtI* gene, introduces four double bonds in the molecule of phytoene to yield lycopene. Finally, the lycopene cyclase, encoded also by the *crtYB* gene, sequentially converts one of the ψ acyclic ends of lycopene as β-ring to form γ-carotene, and subsequently the other to generate β-carotene. Potential biosynthetic steps for the conversion of β- and γ-carotene into xanthophylls in *X. dendrorhous* include the incorporation of two 4-keto groups in the molecule of β-carotene by the ketolase (K) activity, and the introduction of two 3-hydroxy groups by the hydroxylase (H) activity. Both activities (K and H) seem to be included in a single enzyme (astaxanthin synthetase; CrtS) encoded by the *crtS* gene. The cytochrome P450 reductase encoded by the *crtR* gene has an auxiliary role to CrtS, providing with the necessary electrons for substrate oxygenation. The existence of a monocyclic pathway to HDCO has also been proposed (22). Main carotenoids detected in *X. dendrorhous* broths are shown inside a *rectangle*. This figure has been modified from Álvarez et al. (18), with permission.

Attempts to increase astaxanthin content in *X. dendrorhous* involved optimization of cultivation methods and searching for astaxanthin-hyperproducing mutants by screening of classically mutagenized *X. dendrorhous* strains. Additionally, carotenogenic genes have been cloned and transformation systems have been developed, allowing the directed genetic modification of the astaxanthin pathway. However, the knowledge about carotenogenesis regulation is still limited in comparison to other carotenogenic fungi. The cloning and characterization of the genes involved in the astaxanthin biosynthetic pathway of *X. dendrorhous* have provided valuable opportunities to construct improved strains by metabolic pathway engineering. Here, we describe the expression of the cDNA from the *crtS* gene of *X. dendrorhous* in *M. circinelloides* to obtain zeaxanthin overproducing transformants.

2. Materials

This section describes the specific material used in the procedures described in the Subheading 3. Those of general use are only described the first time.

2.1. Flask Fermentation of X. dendrorhous

1. *X. dendrorhous* Y2476 (23).
2. YEPD solid medium (YEPDA): 20 g/L glucose, 10 g/L yeast extract, 20 g/L bacto-peptone, and 20 g/L agar. Adjust pH to 6.0 with HCl. Sterilize at 121°C for 20 min (24) (see Note 1).
3. Saline solution: 0.9 g of NaCl and 100 mL of deionized water. Sterilize at 121°C for 20 min.
4. Seed medium: 70 g/L glucose, 6.2 g/L yeast extract, 5.5 g/L cotton meal, 2 g/L KH_2PO_4, 0.4 g/L K_2HPO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L $(NH_4)_2SO_4$, 0.2 g/L NaCl, 0.2 g/L $CaCl_2$, 50 µg/L biotin, 500 µg/L thiamine HCl, and 2 mg/L calcium pantothenate. Adjust to pH 5.8–6.0 with HCl. Distribute 25 mL aliquots in 500-mL flasks. Sterilize at 121°C for 20 min (25).
5. Fermentation medium: 200 g/L glucose, 6.2 g/L yeast extract, 5.5 g/L cotton meal, 2 g/L KH_2PO_4, 0.4 g/L K_2HPO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L $(NH_4)_2SO_4$, 0.2 g/L NaCl, 0.2 g/L $CaCl_2$, 1.6 g/L $CaCO_3$, 50 µg/L biotin, 500 µg/L thiamine HCl, and 2 mg/L calcium pantothenate. Distribute 25 mL aliquots in 500-mL flasks. Adjust pH to 5.8–6.0 with HCl. Sterilize at 121°C for 20 min (25) (see Note 2).
6. Erlenmeyer flask of 500 mL.
7. Autoclave.

8. Flow laminar cabinet.
9. Freezer –86°C.
10. Milli-Q Integral water purification system (Millipore, Billerica, MA, USA).
11. Elix water purification system (Millipore, Billerica, MA, USA).
12. pH meter.
13. Orbital shaker (see Note 3).

2.2. Cloning of cDNA of crtS Gene from X. dendrorhous by RT-PCR

To avoid RNA degradation: (i) all the material used in this procedure must be RNase-free, (ii) disposable gloves must be used, and (iii) material must be washed with water-DEPC (treated with 0.2% diethyl pyrocarbonate (DEPC) to eliminate RNases) and autoclaved.

2.2.1. Purification of RNA from X. dendrorhous

1. Liquid nitrogen (see Note 4).
2. Mortar and pestle.
3. Centrifuge.
4. Refrigerated centrifuge.
5. RNase-free microtubes.
6. RNeasy plant mini kit (QIAGEN, Valencia, CA, USA).
7. Micropipettes.
8. Agarose gel electrophoresis equipment.
9. TAE (50×) stock solution: Dissolve 242 g of Tris base (MW = 121.14) in approximately 750 mL of ultrapure water. Add 57.1 mL of glacial acid and 100 mL of 0.5 M EDTA, and adjust the solution to a final volume of 1 L with ultrapure water. Sterilize at 121°C for 30 min (see Note 5).
10. TAE buffer (1×) working solution: Dilute the stock solution by 50-fold with sterile ultrapure water. Final concentrations are 40 mM Tris acetate and 1 mM EDTA.
11. Agarose gel (1%): 0.5 g of agarose and 50 mL of 1× TAE buffer (see Note 6).
12. Ethidium bromide (0.5 μg/mL) (see Note 7).

2.2.2. DNA Amplification from RNA by RT-PCR

1. Primer #126 (CTC CCC ATG GTC ATC TTG GTC TTG CTC) (see Note 8).
2. Primer #127 (GAA TCA ACT CAT TCG ACC GGC) (see Note 8).
3. Titan one tube RT-PCR system (Roche Molecular Biochemicals, Mannheim, Germany).
4. PCR thermocycler.
5. Molecular imager gel doc (Bio-Rad, Hercules, CA, USA).
6. QIAex gel extraction kit (QIAGEN, Valencia, CA, USA).

7. pGEM-T (Promega, Madison, WI, USA) (see Note 9).
8. Restriction enzymes.
9. T4-DNA ligase.
10. DNA polymerase I large (Klenow) fragment.
11. *Escherichia coli* DH5α (ATCC, Manassas, VA, USA).
12. Gene pulser II (Bio-Rad, Hercules, CA, USA) (see Note 10).
13. Luria-Bertani (LB) medium: 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, and 20 g/L agar. Adjust to pH 7.0 with 5 N NaOH. Sterilize at 121°C for 15 min.
14. Ampicillin stock solution 100 mg/mL (see Note 11).
15. Kanamycin sulfate stock solution 50 mg/mL (see Note 11).
16. Phenol–chloroform–isoamyl alcohol (25:24:1) (v/v).
17. TE buffer: 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA.
18. IPTG (isopropyl-β-D-1-thiogalactopyranoside).
19. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

2.3. Construction of the Expression Plasmid

1. Plasmid pLeu4 (26) (see Note 12).
2. P*carRA* (27) (see Note 13).

2.4. Transformation of M. circinelloides Protoplasts

1. *B. trispora* F-816 (+) (28) (see Note 14).
2. YPDA: 20 g/L glucose, 20 g/L Bacto-peptone, 10 g/L yeast extract, and 15 g/L agar. Adjust pH to 6.0 with HCl. Sterilize at 121°C for 20 min.

2.4.1. Isolation of Cell Walls of Blakeslea trispora

3. Nytal filter 30-μm pore (Swiss silk bolting cloth fabric, Zurich, Switzerland).
4. Microscope counting chamber (hemocytometer).
5. Ball mill with 0.5-mm grinding beads.
6. Saline solution: 9 g/L NaCl.
7. Sieve (50 mesh, 0.3 mm nominal opening).

2.4.2. Obtaining Chitosanase (Streptozyme) from Streptomyces sp.

1. *Streptomyces* sp. n°6 (29) (see Note 15).
2. Slant tubes (200 × 30 mm).
3. MEY sporulation medium: 4 g/L yeast extract, 10 g/L maltose, 0.5 mL/L $CoCl_2$ (1%), and 20 g/L agar. Sterilize at 121°C for 20 min (30).
4. Jeniaux medium: 5 g/L yeast extract, 3 g/L glucose, 0.5 g/L NaCl, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.01 g/L $FeSO_4 \cdot 7H_2O$. Adjust to pH 7.0 with NaOH 30%. Sterilize at 121°C for 20 min (31).
5. Induction medium: 20 g/L cell walls from *B. trispora*, 0.5 g/L NaCl, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.01 g/L $FeSO_4 \cdot 7H_2O$. Sterilize at 121°C for 20 min.

6. Orbital shaker (see Note 16).
7. Bench centrifuge.
8. PG buffer: 4.82 g/L $NaH_2PO_4 \cdot H_2O$ and 4.03 g/L of $Na_2HPO_4 \cdot 7H_2O$ in a 1 L of deionized water. Add 50 g glycerol and mix until homogenization. Check the pH before sterilize at 121°C for 20 min.
9. PD-10 desalting columns (GE Healthcare, Miami, FL, USA) (see Note 17).
10. Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA).

2.4.3. Obtaining and Transformation of Protoplasts from M. circinelloides

1. *M. circinelloides* MS12 (–) (*leuA1*, *pyrG4*) (32) (see Note 18).
2. YPG: 20 g/L glucose, 3 g/L yeast extract, 10 g/L Bacto-peptone, and 6 g/L malt extract. Adjust to pH 4.5. Sterilize at 121°C for 20 min (33).
3. Uracil (St Louis, MO, USA) (see Note 19).
4. Leucine (St Louis, MO, USA) (see Note 19).
5. Sodium phosphate buffer 10 mM pH 6.5: 0.964 g of $NaH_2PO_4 \cdot H_2O$ and 0.807 g of $Na_2HPO_4 \cdot 7H_2O$ in 1 L of deionized water. Check the pH and sterilize at 121°C for 20 min.
6. Caylase C3 (Cayla, Toulouse, France) (see Note 20).
7. YNB: 0.5 g/L yeast nitrogen base without amino acids, 1.5 g/L ammonium sulfate, 10 g/L glucose, 1.5 g/L glutamic acid, and 20 g/L agar. Sterilize at 121°C for 20 min (34) (see Note 21).
8. Soft YNB: YNB with 10 g/L agar (instead of 20 g/L). Sterilize at 121°C for 20 min (see Note 21).
9. SMC buffer: 0.55 M sorbitol, 10 mM MOPS pH 6.3, and 50 mM $CaCl_2$. Sterilize at 121°C for 20 min.
10. PMC buffer: 40% PEG 4000, 10 mM MOPS pH 6.3, 0.4 M sorbitol, and 50 mM $CaCl_2$. Sterilize at 121°C for 20 min.

2.5. Carotenoid Analysis

1. Dichloromethane:methanol (50:50).
2. Alliance high performance liquid chromatography (HPLC) equipment, including the 2960, separation module and 996 PAD (Waters, Milford, MA, USA).
3. Nucleosil 100 NH_2 column (resin particle diameter, 5 μm; 250 × 4.6 mm).
4. Mobile phase: Hexane:ethyl acetate (50:50).
5. Zeaxanthin standard solution (Sigma, St Louis, MO, USA) (see Note 22).

3. Methods

This section describes the heterologous expression of *crtS* gene from *X. dendrorhous* in *M. circinelloides* to produce xanthophylls. The methods outline the flask fermentation of *X. dendrorhous* to produce astaxanthin, the cloning of the cDNA of *crtS* gene by RT-PCR from RNA of *X. dendrorhous* obtained in astaxanthin production conditions the construction of an expression plasmid of this cDNA under the control of the P*carRA* promoter of *B. trispora*, the selection of *M. circinelloides* transformants, and the carotenoid profile of these transformants. DNA manipulations are performed according to standard procedures (35).

3.1. Flask Fermentation of X. dendrorhous

Fermentation of *X. dendrorhous* is carried out as previously described (25), with some modifications.

1. Inoculate 1 mL of frozen cells of *X. dendrorhous* Y2476 in a Petri plate (15-cm diameter) with 75 mL of YEPDA and incubate for 120 h at 17–20°C.
2. Resuspend the cells in 1.5 mL of sterile saline solution and seed a 500-mL flask with 25 mL of seed medium (see Note 23).
3. Incubate at 17–20°C and 250 rpm for 48 h.
4. Seed 2.5 mL of seed culture into a 500-mL flask with 25 mL of fermentation medium. Incubate for 120–168 h at 17–20°C and 250 rpm under illumination.

3.2. Cloning of the crtS cDNA from X. dendrorhous by RT-PCR

One of the limitations of the heterologous expression of eukaryotic genes is the capability of the heterologous host for the proper processing of introns. The *crtS* gene from *X. dendrorhous* has 17 introns, which largely difficult their correct processing by the host. The expression of the cDNA solves this problem as it has spliced introns. This section describes the purification of total RNA from *X. dendrorhous* in astaxanthin production conditions to maximize the *crtS* expression (Subheading 3.2.1), and cDNA amplification by RT-PCR for further expression (Subheading 3.2.2).

3.2.1. Purification of RNA from X. dendrorhous

RNA from *X. dendrorhous* is isolated using the RNeasy plant mini kit, a technology that combines the selective binding properties of a silica gel-based membrane with the speed of microspin technology. This procedure isolates high-quality RNA molecules longer than 200 nucleotides, showing a size distribution of the isolated RNA comparable to that obtained by centrifugation through a CsCl cushion.

1. Centrifuge the *X. dendrorhous* culture at 4,000 × *g* for 10 min at 4°C and carefully recover the cells.

2. Freeze immediately the cells in liquid nitrogen, and store at −70°C until use (see Note 4).
3. Disrupt the cells in a mortar under liquid nitrogen to obtain a fine powder (see Note 24).
4. Transfer a quantity of powder of about 500 μL to a liquid nitrogen-cooled sterile microtube (2.2 mL) with the help of a frozen spatula (see Note 25).
5. Purify RNA using RNeasy plant mini kit.
6. Analyze 3–5 μL of RNA sample onto 1% agarose gel and perform electrophoresis at 120 V for 20 min (see Note 26).

3.2.2. RT-PCR

This procedure allows synthesis of cDNA from mRNA and subsequent amplification of the DNA. In this case, we use the "Titan One Tube RT-PCR System" according to the instructions of the manufacturer. All the material must be RNase-free to avoid sample degradation. Disposable gloves must be used and material must be autoclaved twice. Solutions must be done with water treated with 0.2% diethyl pyrocarbonate (water-DEPC) to eliminate RNases.

1. Thaw the RNA samples in an ice water bath.
2. Prepare the RT reactions with the reactives from the "Titan One Tube RT-PCR System."
3. Incubate RNA with dNTPs, primer #126, primer #127, AMV reverse transcriptase, and DNA polymerase at 50°C for 30 min.
4. Transfer the samples to a thermocycler and do 35 cycles of amplification reactions as follows: 94°C, 10 s; 58°C, 30 s; 68°C, 60 s (extending 5 s per cycle after 11th).
5. Load the sample onto 0.7% agarose gel and perform electrophoresis at 100 V for 40 min (see Note 27).
6. Gel purify the DNA fragment using "Qiaex II Gel Extraction Kit," and resuspend the DNA in 10 μL of sterile ultrapure water.
7. Check 1 μL DNA by 0.7% agarose gel electrophoresis (100 V for 40 min).
8. Digest pGEM-T with *Not*I and fill the edges with DNA polymerase (Klenow). Then, digest with *Nco*I and ligate to the PCR fragment previously digested with *Nco*I.
9. Check the cloned fragment in pGEM-T by sequencing analysis to confirm the absence of mutations that may have occurred during the DNA amplification from RNA.

3.3. Construction of an Expression Plasmid

The autonomous replicating plasmid pALMC11 is constructed to express the cDNA of the *crtS* gene of *X. dendrorhous* in *M. circinelloides* MS12. This plasmid includes the *crtS* cDNA (1.6 kb *Nco*I–*Not*I) expressed under the control of the *carRA* promoter of *B. trispora* (611 pb *Nco*I) (27), and the *leuA* gene as an auxotrophic marker (26)

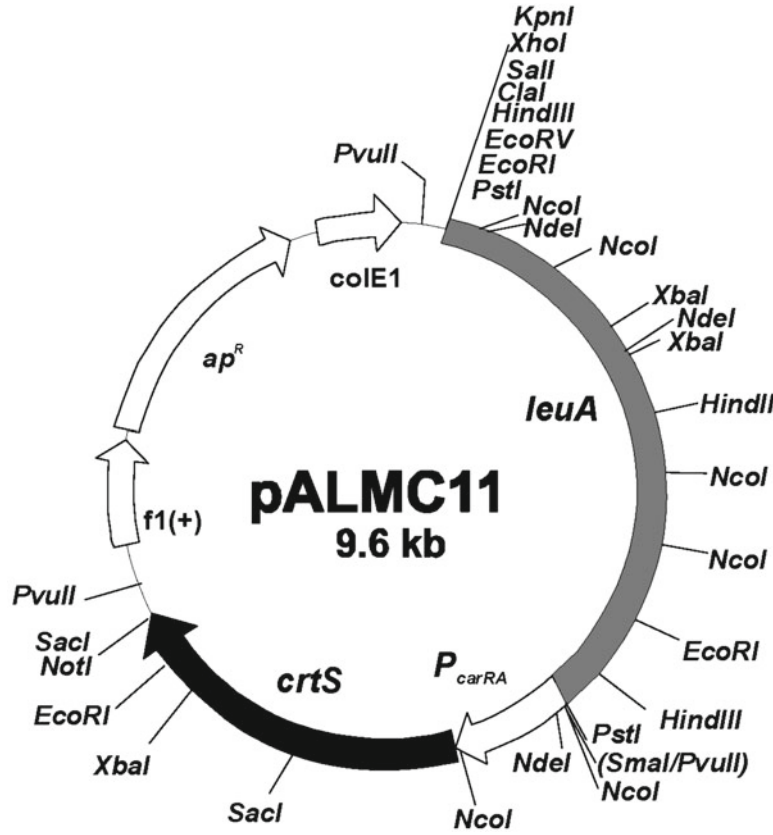

Fig. 2. Restriction map of the plasmid pALMC11 used for the expression of the cDNA from the *crtS* gene of *X. dendrorhous* in *M. circinelloides* MS12. This figure has been reproduced from Álvarez et al. (18), with permission.

(Fig. 2). General procedures for plasmid DNA purification, cloning, and transformation of *E. coli* are those of Sambrook et al. (35).

1. To digest the DNA, mix 1–3 μg of DNA, 5 μL of 10× buffer, and 25–40 U of enzyme adding ddH_2O to a final volume of 50 μL. Incubate at 37°C for 3 h.
2. Check the digestion products by 0.7% agarose gel electrophoresis (120 V for 20–30 min).
3. Purify the digestion products with the "Qiaex II Gel Extraction Kit."
4. Resuspend the purified DNA in 10 μL of sterile ultrapure water for further reactions.
5. Prepare standard ligation reactions (35).

3.4. Transformation of M. circinelloides Protoplasts

One of the most efficient methods to express exogenous DNA in zygomycete fungi is the polyethylene glycol-based transformation method. This procedure requires the previous formation of protoplasts to allow introducing the exogenous DNA through the cell membrane. The cellular wall of Zygomycetes fungi as *M. circinelloides* is mainly composed of complex polysaccharides such as chitin and chitosan that confers a strong rigidity to the cell. The treatment with lytic enzymes able to digest this type of structure results in the formation of protoplasts, susceptible then for DNA transformation. Here we describe the protocol to obtain Streptozyme (31), a lytic enzymatic mixture synthesized by *Streptomyces* sp. n°6 in the presence of cellular walls of fungi, able to degrade chitosan. In this procedure, we use cellular walls from *B. trispora*, a β-carotene producing Mucoral fungus, to induce the synthesis of the lytic enzymes (Subheadings 3.4.1 and 3.4.2). Transformation of *M. circinelloides* is carried out as previously described (36), with some modifications (Subheading 3.4.3).

3.4.1. Isolation of Cell Walls of B. trispora

1. Seed about 10^7 spores of *B. trispora* F-816 (+) on Petri plates with 75 mL of YPDA medium.
2. Incubate for 3–5 days at 25°C until the aerial mycelium covers the surface of the plates.
3. Recover carefully the mycelium with a sterile spatula and wash three times with sterile water by centrifugation at 3,000 × *g* and room temperature (see Note 28).
4. Freeze the mycelium at –20°C for several hours and then thaw to room temperature (see Note 29).
5. Disrupt the mycelium using a ball mill by ten cycles of 2 min at 4°C, leaving 30 s among each cycle (see Note 30).
6. Filter through 50-mesh sieve to recover the grinding beads (see Note 31).
7. Centrifuge the filtrate at 7,000 × *g* for 15 min.
8. Wash the pellet with 100 mL of sterile water and centrifuge again in the same conditions.
9. Repeat the washing steps until the supernatant is transparent.
10. Transfer the pellet to a tube and weigh it. This extract is used at final concentration of 2% to prepare the induction medium.

3.4.2. Obtaining of Chitosanase Enzyme (Streptozyme) from Streptomyces sp.

1. Prepare slant tubes with 25 mL of MEY (see Note 32).
2. Seed the slant tubes with 0.1 mL of frozen spores of *Streptomyces* sp. n°6, and incubate for 7 days at 28°C until sporulation.
3. Resuspend the spores of each slant in 10 mL of sterile water, briefly scraping with a sterile pipette.
4. Seed 0.25 mL of the spore solution in 500-mL flasks with 75 mL of Jeniaux medium. Incubate at 28°C and 250 rpm for 48 h.

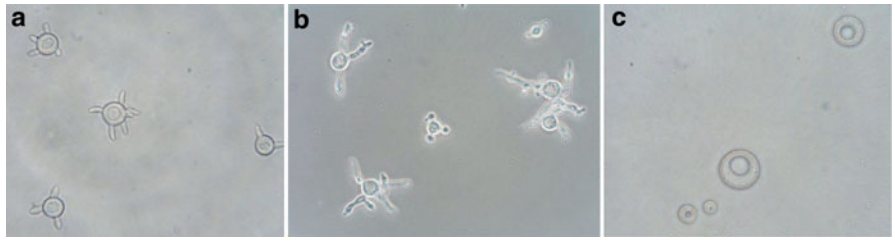

Fig. 3. Microscopic observation of the formation of *M. circinelloides* protoplasts (×40). (**a**) Early germination of spores. (**b**) Germinated spores ready for lytic enzyme treatment. (**c**) Protoplasts.

5. Harvest the mycelium by centrifugation at 4,000 × *g* for 5 min in sterile conditions. Wash the pellet with sterile water and centrifuge in the same conditions.
6. Resuspend the pellet in 1/10 volume of sterile water and seed 5 mL in 500-mL flasks with 75 mL of induction medium. Incubate at 28°C and 250 rpm for 18–24 h.
7. Centrifuge the culture at 7,000 × *g* for 30 min at 4°C and recover the supernatant.
8. Precipitate with ammonium sulfate using 50 g of salt for each 100 mL supernatant (see Note 33).
9. Centrifuge at 10,000 × *g* for 30 min at 4°C. Discard the supernatant and resuspend the pellet in the minimum volume of PG buffer.
10. Equilibrate PD-10 desalting columns with 4 volumes of PG buffer.
11. Add 2 mL of sample and elute with PG buffer, harvesting 2 mL aliquots in sterile tubes (see Note 34).
12. Add cold glycerol to a final concentration of 20% and freeze the aliquots at −20°C until their quantification.
13. Check the protein content by the Bradford method (37) using the "Bio-Rad Protein Assay."

3.4.3. Transformation of M. circinelloides

1. Inoculate *M. circinelloides* MS12 at about 10^7–10^9 spores/mL in flasks of 100 mL with 10 mL of YPG medium supplemented with 0.2 g/L of leucine and 0.4 g/L of uracil.
2. Incubate for 2 h at room temperature without agitation and 12 h at 4°C. Then, incubate at 28°C and 200 rpm to favor spore germination.
3. Take samples after 2.5–3 h incubation and check the germination rate by microscopic examination (Fig. 3).
4. Once germinated, centrifuge the culture at 1,000 × *g* for 5 min at 4°C in sterile conditions. Wash the pellet with 10 mM phosphate buffer pH 6.5 and centrifuge again.

5. Resuspend the pellet in 10 mL of 10 mM phosphate buffer pH 6.5 supplemented with 0.55 M sorbitol.
6. Add the lytic enzymes: 1.5 mg/mL of caylase C3 and 0.5 mL of streptozyme (Subheading 3.4.2).
7. Mix homogenously and incubate in a 100-mL flask at 22°C for 2–3 h with gentle agitation (no more than 65 rpm).
8. Check the protoplast release by microscopic examination (see Note 35).
9. Centrifuge the protoplast suspension at 4,000 × *g* for 5 min at 4°C.
10. Discard the supernatant and carefully wash the protoplast pellet twice with SMC buffer, by centrifugation at 4,000 × *g* for 5 min at 4°C.
11. Suspend the pellet in 1–2 mL of SMC buffer and check the protoplast concentration by microscopic observation.
12. Mix 200 μL of protoplast suspension with 0.5–5 μg of the expression plasmid pALMC11 and 20 μL of PMC buffer in a 10-mL sterile polypropylene tube.
13. Incubate for 30 min in a water-ice bath and then add 2.5 mL of PMC buffer, mixing gently by inversion until homogenization. Incubate at room temperature for 25 min.
14. Wash with 10 mL of SMC buffer and centrifuge at 4,000 × *g* for 5 min at 4°C.
15. Resuspend in 5 mL of YPG medium supplemented with 0.55 M sorbitol pH 4.5 and incubate with gentle agitation (50–65 rpm) for 30 min at room temperature.
16. Wash twice with 10 mL of SMC buffer as indicated in step 10 and finally resuspend in 3 mL.
17. Add 6 mL of soft YNB medium, mix gently inverting the tube, and spread over Petri plates with YNB medium supplemented with 0.4 g/L uracil (see Note 36).
18. Incubate for 4–5 days at 22°C until the colonies appear, and transfer them to individual fresh plates for storage and further analysis.

3.5. Verification of the Transformants

1. Confirm the stability of the transformants by successive growth cycles in YNB medium supplemented with 0.4 g/L of uracil (see Note 37).
2. Inoculate the selected colonies into 10 mL of liquid YNB medium supplemented with 0.4 g/L of uracil. Grow at 30°C for 3–5 days with 250 rpm agitation.
3. Mix 5 mL of culture with 5 mL of 50% glycerol, and aliquot in cryogenic vials for their conservation. Store at −70°C until use.

4. Use the remaining culture to purify plasmid DNA.
5. Mix 2 μL of isolated plasmid with 50 μL of *E. coli* DH5α cells and put the mixture into a prechilled electroporation cuvette.
6. Electroporate the cells at 2.5 kV, and quickly add 1 mL of LB medium.
7. Incubate at 37°C and 250 rpm for 1 h, and then spread the cells on a LB plate supplemented with 100 μg/mL ampicillin.
8. Incubate the Petri plates at 37°C for 16–18 h until colonies appear.
9. Inoculate single colonies in 3 mL of LB supplemented with 100 μg/mL ampicillin, and grow at 37°C for 12–16 h.
10. Purify the plasmids from each culture.
11. Verify the integrity and lack of rearrangements of pALMC11.

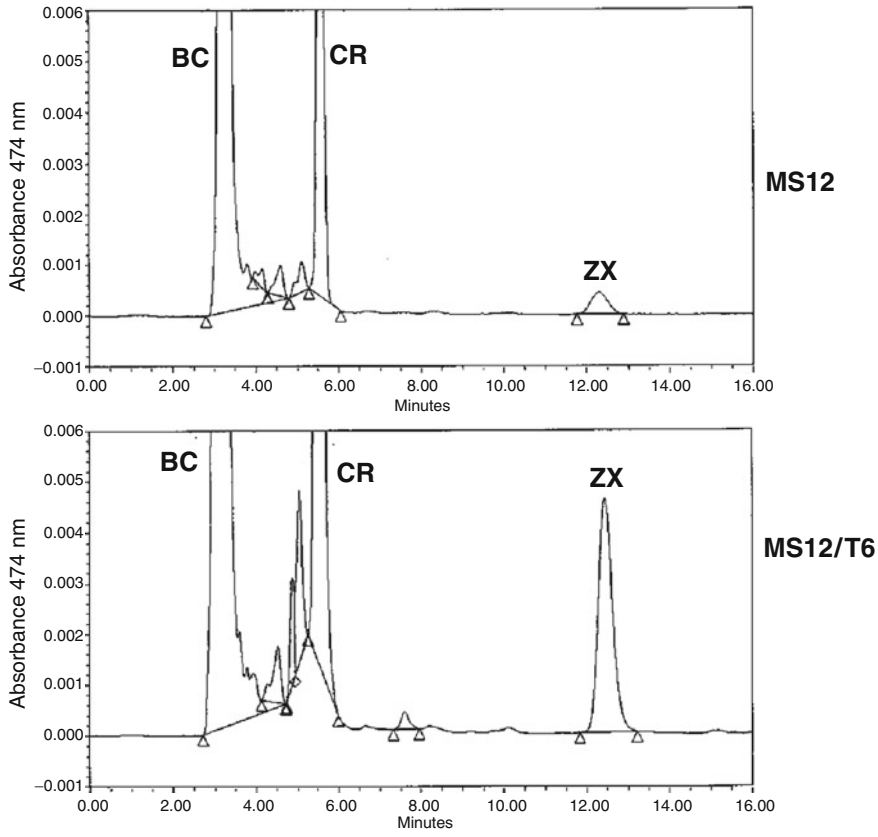

Fig. 4. HPLC analysis of carotenoids produced *by M. circinelloides* MS12 and the transformant MS12/T6. *M. circinelloides* carotenoid production was tested using as starting material the same quantity of mycelium (0.5 g) grown on YNB plates. β-carotene (BC), β-cryptoxanthin (CR), and zeaxanthin (ZX). This figure has been reproduced from Álvarez et al. (18), with permission.

3.6. Zeaxanthin Analysis

1. Place 1 g of total broth in a 100-mL flask and extract with methanol/dichloromethane (50/50, v/v) for 1 h under magnetic stirring.
2. Filter through nylon—0.2 μm and dilute with acetone if necessary.
3. Perform HPLC analysis at 474 nm using Nucleosil 100 NH_2 column and hexane/ethyl acetate (50/50 ratio) at 1 mL/min flow rate as mobile phase (Fig. 4).

4. Notes

1. To favor the complete dissolution of the agar, prepare the medium with hot water and under stirring.
2. Glucose must be autoclaved separately to avoid Schiff base reaction during the sterilization.
3. For optimal astaxanthin productivity, the orbital shaker should work at 250 rpm with 5 cm stroke under illumination. Astaxanthin biosynthesis is induced by white or UV light in *X. dendrorhous* (23, 28). Best results are obtained with three-baffled, high collar 500-mL flasks, and using milk filters instead foam plugs to allow maximum aeration.
4. Liquid nitrogen must be manipulated with caution. Skin contact may lead to a frostbite burn. The release of large quantities of liquid nitrogen in a confined space displaces air and can lead to asphyxiation.
5. This stock solution can be stored at room temperature. The pH of this buffer is not adjusted and should be about 8.5.
6. Microwave until agarose is completely melted. Cool the solution to approximately 70–80°C. Wash the electrophoresis equipment and the comb with 10% hydrogen peroxide and rinse with ultrapure water. Pour 25–30 mL of solution onto an agarose gel rack with appropriate 2- or 8-well combs.
7. Ethidium bromide is a carcinogen and must be manipulated with caution. Wear gloves and discard the contaminated tips and gels in specific containers for ethidium bromide solid waste disposal.
8. Primers are designed from nucleotide sequence of the *crtS* gene (39). Primer #126 includes a target site for the restriction endonuclease *Nco*I (underlined) to facilitate the subsequent cloning of the amplified DNA in different expression vectors.
9. pGEM-T is used for subcloning of PCR products. It has a size of 3,006 bp and confers resistance to ampicillin.

10. Gene pulser II is used to transform plasmids into *E. coli* by electroporation.
11. Ampicillin and kanamycin are antibiotics used for the selection of plasmid transformants. They are dissolved in water and sterilized by filtration. Final volumes of the stock solutions added to the culture medium are 100 μL/mL ampicillin and 50 μL/mL kanamycin.
12. Plasmid pLeu4 is derived from pUC13, which includes a 4.4-kb *Pst*I fragment with the *leuA1* gene for the complementation of the leucine auxotrophy (leu-). It also has the *ampR* gene that confers resistance to ampicillin.
13. It is a 611-bp *Nco*I fragment that includes the *carRA* promoter of *B. trispora*.
14. *B. trispora* F-816 (+) is a β-carotene overproducing strain. Its cell walls are used to induce chitosan lytic enzymes in *Streptomyces* sp. n°6.
15. This *Streptomyces* strain is used to obtain lytic enzymes able to degrade the chitosan of the *M. circinelloides* cell walls.
16. Optimal working conditions are 250 rpm and 5 cm stroke.
17. PD10 desalting columns containing 8.3 mL Sephadex™ G-25 resin for separation of high (Mr > 5,000) and low molecular weight substances (Mr < 1,000) by desalting and buffer exchange.
18. *M. circinelloides* MS12 (−) is an auxotrophic strain for leucine and uracil used for heterologous expression of *crtS* gene of *X. dendrorhous*.
19. Uracil and leucine are amino acids used to complement strains auxotrophies. Uracil is used at a final concentration of 400 mg/L of culture medium, and leucine at 200 mg/L.
20. Caylase C3 is an enzymatic mixture from *Tolypocladium geodes*.
21. Adjust YNB to pH 3.5 to grow isolated colonies or to pH 4.5 for a spread growth.
22. Dissolve 10 mg of zeaxanthin in 500 mL of hexane:ethyl acetate (50:50). Take 5 mL and dilute to 50 mL with acetone. Aliquot in vials and freeze at −20°C. To check the zeaxanthin concentration, measure OD_{452} with a specific extinction coefficient of 2,340 (100 mL/g/cm) (40).
23. Use crystal pipettes because *X. dendrorhous* is readily adsorbed on polystyrene surfaces.
24. Before to put the frozen cells in the mortar, clean the mortar with ethanol and cool it with liquid nitrogen.
25. Allow to evaporate the liquid nitrogen avoiding thawing the sample. Spatula must be cooled.

26. TAE 1× must be prepared with RNase-free water and then autoclaved to eliminate RNases. Electrophoresis equipment must be cleaned with diluted H_2O_2.
27. The size of amplified fragment must match the predicted by sequence. In this case, the amplified fragment of 1674 bp corresponds to the size of the *crtS* gene after introns processing.
28. Filtration through Nytal filter (30-μm pore) can be done instead the washing steps.
29. The freezing and thawing cycle promotes the cell wall disruption process.
30. To avoid overheating, refrigerate previously the mill by circulating cold water for 15 min. Use a grinding beads/mycelium ratio of 7/10 (v/v).
31. The glass grinding beads can be recycled by washing with water, 24 h treatment with concentrated sulfuric acid, and washing again with water to neutral pH.
32. The slants must be incubated before seeding for 2 days at 28°C. This dryness of the medium surface promotes *Streptomyces* sporulation.
33. Finely mill ammonium sulfate in a mortar to favor its dissolution. Mix at 4°C for 5 min maximum, controlling the pH and avoiding foaming. Then, stand at 4°C for 2–3 h before centrifugation.
34. If the sample volume after suspension is high, the desalting step can be achieved by overnight dialysis through a 5,000 Da

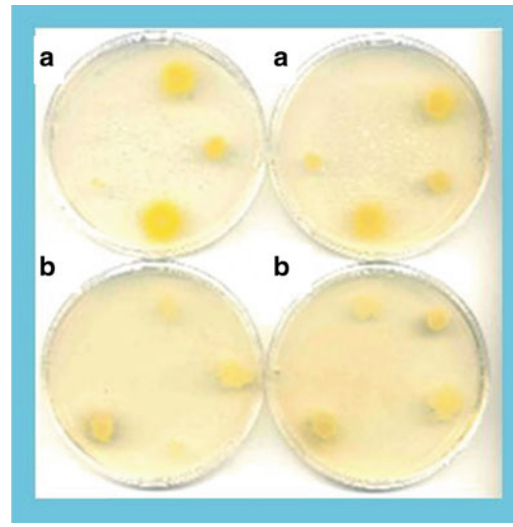

Fig. 5. Isolated transformants of *M. circinelloides* MS12. (**a**) Transformants including pALMC11. (**b**) Transformants including the expression plasmid without *crtS* gene (negative control).

cutoff membrane in PG buffer. In this case the cocktail activity expected will be lower due to higher volume and autolysis.

35. The protoplast yield after 3 h incubation should be more than 70%. If the protoplast yield is low, try to reduce the seeding, to reduce the germination time, to increase the washing volume, or to increase a bit the lytic enzyme concentration. Do not increase the lysis time (burst danger).
36. The growth of *M. circinelloides* in solid medium is very fast (3–6 cm/day). Lowering pH allows growing *M. circinelloides* as isolated colonies.
37. The transformants expressing pALMC11 show more intense yellow pigmented mycelium than the parental strain without the *crtS* cDNA (Fig. 5).

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