Chapter 12

Isolation and Selection of New Astaxanthin Producing Strains of *Xanthophyllomyces dendrorhous*

Diego Libkind, Martín Moliné, and Celia Tognetti

Abstract

Astaxanthin is a xanthophyll pigment of high economic value for its use as a feeding component in aquaculture. *Xanthophyllomyces dendrorhous* is a basidiomycetous fungi able to synthesize astaxanthin as its major carotenoid, and the only known yeast species bearing the capability to produce this type of carotenoid. Recently, the habitat and intraspecific variability of this species have been found to be wider than previously expected, encouraging the search for new wild strains with potential biotechnological applications. Here we describe effective procedures for isolation of *X. dendrorhous* from environmental samples, accurate identification of the strains, analysis of their astaxanthin content, and proper conservation of the isolates.

Key words: Astaxanthin, Phaffia, Xanthophyllomyces, ITS, Selective culture media, Natural environments, Mycosporines, Sequencing

1. Introduction

The ability of the basidiomycetous yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) to accumulate the antioxidant, astaxanthin, as its major carotenoid is responsible for the industrial use of this yeast as a microbial source of pigments for aquaculture (1). Astaxanthin is economically important because it is the most expensive aquaculture feed component (2); it is used for the artificial pigmentation of fish and crustaceans (3). Astaxanthin is, in fact, one of the most expensive components of salmon farming, accounting for about 15% of total production costs (4).

Original isolations of *X. dendrorhous*, which have served for most of the studies involving this species, were carried out in the 1960s by Phaff et al. (5). These isolates were obtained from slime exudates of various broad-leaved trees in different mountainous regions in the northern hemisphere, such as Japan and Canada.

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More recently, isolations of this species have been carried out in Italy (6), Germany (7), Argentina (8), and the USA (9).

The isolations carried out in Argentina (NW Patagonia) were the first to be reported in the southern hemisphere, and unlike all previous isolations, they were not from sap flow, but rather from ascostroma of *Cyttaria hariotii* (8) parasiting the tree *Nothofagus dombeyi*. A few isolates were also obtained from a mountain lake. Patagonian strains of *X. dendrorhous* bear genetic differences with collection *X. dendrorhous* strains. These differences have been hypothetically explained by geographic isolation and habitat specificity (8). Even more recently, a new astaxanthin producing yeast isolate was obtained from a leaf of *Eucalyptus globulus* in Chile (10). This strain is remarkably different from all previous strains regarding the rRNA genes containing the internal transcribed spacers (ITS) sequences. These differences probably imply that the Chilean strain represents a novel species in the genus *Xanthophyllomyces*.

Thus, it becomes clear that the natural geographic distribution of *Xanthophyllomyces* is wider than expected, and that its habitat is not restricted to slime fluxes, but rather, includes sugary rich fungal stromata, leaves, and freshwater. Furthermore, the genetic variability of this astaxanthin producing yeast is also more diverse than what was previously thought, and it is likely that there is more than one species in the genus. The biotechnological potential of the recently discovered Patagonian isolates has only been preliminary assessed (11).

In this chapter, we describe the strategies currently used in our laboratory for the effective isolation of *Xanthophyllomyces* sp. from environmental samples, the accurate identification of the strains, the analysis of their astaxanthin content, and the proper conservation of the isolates.

2. Materials

Prepare all solutions using distilled sterile water and analytical grade reagents. Prepare and store all reagents at room temperature (unless otherwise indicated). Follow all waste disposal regulations when disposing waste materials.

2.1. Isolation of X. dendrorhous from Environmental Samples

1. YM agar: 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L dextrose, and 20 g/L agar. Adjust pH 4.5–5.0 and supplement with 200 mg/L chloramphenicol.
2. YPD agar: 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar. Adjust pH 4.5–5.0 and supplement with 200 mg/L chloramphenicol.

3. 0.9% NaCl.
4. Filter membranes (0.45 μm) (Millipore Corporation, Bedford, MA, USA).
5. Sterile filtering apparatus.

2.2. Identification of Wild X. dendrorhous Isolates Using Phenotypic Characteristics

1. YM agar (see Subheading 2.1).
2. Lugol's iodine: 0.33% w/v iodine (I_2) and 0.66% w/v potassium iodide (KI) in distilled water, to make a brown solution with a total iodine concentration of 150 mg/mL.
3. Fermentation base medium: 4.5 g/L yeast extract, 7.5 g/L peptone, 20 g/L glucose, and enough Bromothymol blue to obtain a sufficiently dark green color.
4. Durham tubes.
5. Illumination apparatus emitting visible light (photosynthetically active radiation) with or without UVA (see Note 1).
6. 25% v/v Methanol solution.
7. UV–visible spectrophotometer, with quartz cuvettes.
8. Thermostatic bath.
9. Incubation chamber with controlled temperature (20°C).
10. Ribitol agar: 5 g/L ribitol and 20 g/L agar.

2.3. Molecular Identification of Wild X. dendrorhous Isolates

1. YM agar (see Subheading 2.1).
2. ITS5 forward primer: 5′-GGA AGT AAA AGT CGT AAC AAG G-3′.
3. LR6 reverse primer: 5′-CGC CAG TTC TGC TTA CC-3′.
4. NL1 forward primer 5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′.
5. NL4 reverse primer 5′-GGT CCG TGT TTC AAG ACG G-3′.
6. ITS1 forward primer 5′-TCC GTA GGT GAA CCT GCG G-3′.
7. ITS4 reverse primer 5′-TCC TCC GCT TAT TGA TAT GC-3′.
8. PCR mix kit.
9. Thermocycler.
10. Genomic DNA extraction kit.
11. Lysing buffer: 50 mmol Tris-HCL, 250 mmol NaCl, 50 mmol EDTA, and 0.3% w/v SDS, pH 8.
12. PCR product purification kit.

2.4. Extraction, Detection, and Quantification of Astaxanthin

1. C18 HPLC column (5 µm, 250×4.6 mm).
2. Acetonitrile/methanol/isopropanol: 40 mL acetonitrile, 50 mL methanol, and 10 mL isopropanol.
3. Astaxanthin standard (Sigma, St Louis, MO, USA).
4. Carotenoid extract from a reference *X. dendrorhous* collection strain (e.g., CBS 7918^T) (12).

2.5. Preservation of X. dendrorhous Wild Strains

1. YM agar (see Subheading 2.1).
2. Ultrafreezer (−80°C).
3. 20% Glycerol sterile solution.
4. Sterile 425–600 µm Ø glass beads.

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1. Isolation of X. dendrorhous from Natural Environments

To date, *X. dendrorhous* has been found in association to different types of natural substrates, including slime exudates, fungal stromata, leaves and, less frequently, in water samples. Slime exudates are typically found during the spring on birch or beech stumps, and when colonized by *X. dendrorhous*, have an orange-characteristic color. Photos of fluxes colonized by *X. dendorhous* can be seen in Weber et al. (7). Such fluxes have so far only been found in the northern hemisphere in tree species of the genera *Betula*, *Cornus*, *Carpinus*, and *Fagus*. In contrast, there are no reports of the presence of this yeast in exudates of trees from the south hemisphere. However, *X. dendrorhous* has been found in the stromata of the *Nothofagus* spp. parasitic fungus *Cyttaria*, and less frequently in leaves (*Nothofagus* and *Eucalyptus*) and freshwater samples (8, 10). In this subheading, we provide hints for an effective isolation of this yeast species from the different substrates, each of which require a specific procedure to free yeast cells.

3.1.1. Isolation of X. dendrorhous from Exudate Samples

1. Collect exudate samples aseptically and streak out directly onto agar plates.
2. Alternatively, plate out 0.1 mL aliquots of a dilution series obtained by shaking 1 g (fresh weight) of exudate in 10 mL 0.9% sterile aqueous NaCl or distilled water, and stepwise dilution of the resulting suspension in 0.9% NaCl or distilled water.

3.1.2. Isolation of X. dendrorhous from Stromata Samples

1. Collect aseptically stromata of *C. hariotii*, an ascomycetous fungus parasite of *N. dombeyi* ("Coihue").
2. Place them in sterile plastic bags with a known volume of distilled sterile water (5–50 mL).
3. Crush manually *Cyttaria* fruit bodies through the plastic bag and agitate in an orbital shaker for 30 min at 300 rpm.
4. Dilute the extract conveniently and spread aliquots on agar plates.

3.1.3. Isolation of X. dendrorhous from Leaves Samples

1. Collect aseptically whole leaves (as fresh as possible).
2. Place leaves (1–15 depending on size) in a 10- to 15-mL Falcon tube with 8–10 mL distilled water (just enough to cover leaves).
3. Vortex vertically at maximum speed for 5 min.
4. Shake samples overnight at 150 rpm in an orbital shaker.
5. Dilute conveniently the extract.
6. Spread aliquots on agar plates.

3.1.4. Isolation of X. dendrorhous from Water Samples

1. Collect water samples in sterile flasks.
2. Filter them preferably in situ (<15 in. of mercury) through filter membranes (0.45 μm) with a sterile filtering apparatus.
3. Place the filters directly on agar plates.

3.1.5. Isolation of X. dendrorhous by Enrichment

This is an additional isolation method for yeasts that may be applied to any of the previous substrates.

1. Incubate small samples in shaken flasks (100 mL liquid medium with antibiotics) in an orbital shaker (120 rpm) for 48 h.
2. Filter through a sterile glass wool.
3. Do dilution series in fresh medium.
4. Plate out on agar plates.

Recommended temperature range for incubation of plates is 15–20°C, with a 17°C optimum. Normally, 5 days of incubation are enough for colony emergence (see Note 2). Transfer orange colonies to YM or YPD fresh medium and purify.

3.2. Identification of Wild X. dendrorhous Using Phenotypic Characteristics

X. dendrorhous possess such unique morphological and physiological characteristics that makes identification based on phenotypic traits a viable alternative. Even if not used as only mean of identification, the tests described in this subheading are useful for reducing the number of putative *X. dendrorhous* isolates to the minimum. The distinctive combination of phenotypic traits includes: ability to produce amyloid compounds, glucose fermentation capabilities (13), mycosporine production (Fig. 1) (14),

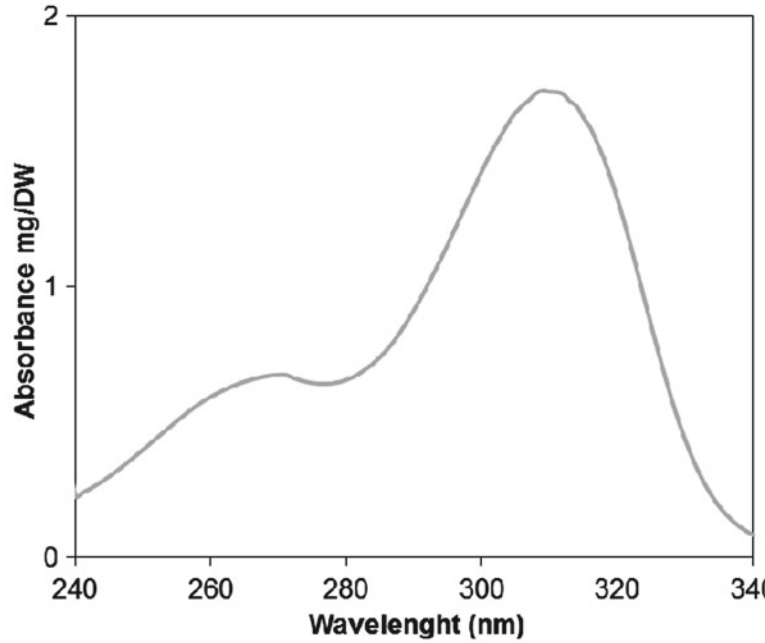

Fig. 1. UV–visible spectrum of mycosporine-glutaminol-glucoside in X. dendrorhous extract. DW dry weight.

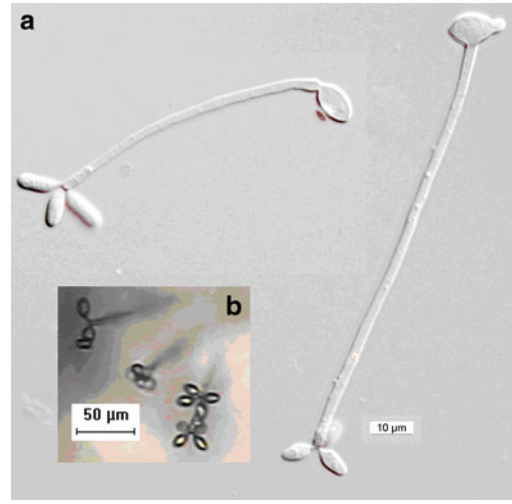

Fig. 2. Sexual structures of *X. dendrorhous*. (**a**) Daughter cell–mother cell conjugation (pedogamy) and holobasidium with terminal basidiospores. (**b**) Terminal basidiospores.

development of unique sexual structures (Fig. 2) (12), and synthesis of astaxanthin as the main carotenoid pigment (15). Any one of these features can be tested to eliminate non-*X. dendrorhous* isolates and the combination of all of these features will provide an accurate identification.

3.2.1. Production of Starch Compounds

1. Grow wild isolates on YM agar for 1 week at 20°C.
2. Flood the plates with five times diluted Lugol's iodine (see Note 3).

3.2.2. Glucose Fermentation

1. Dispense 5–6 mL of base fermentation medium into 150× 12 mm plugged test tubes containing a small inverted tube (Durham tube), approximately 50 mm high and 6 mm Ø.
2. Inoculate with 100 μL of a dense cell suspension of the yeast isolate.
3. Incubate for 1 week at 20°C (see Note 4).

3.2.3. Mycosporine Production

1. Grow yeast isolates on YM agar plates under illumination conditions for 3 days (see Note 1).
2. Harvest biomass and transfer to Eppendorf tube containing 1 mL of 25% (v/v) methanol.
3. Incubate in a water bath at 45°C for 2 h.
4. Clear extracts by centrifugation at 16,000 × *g* for 10 min.
5. Obtain full spectrum within the range 260–400 nm in an UV–visible spectrophotometer using quartz cuvettes.
6. Compare with reference strain or with Fig. 1.

3.2.4. Sexual Structure Formation

1. Inoculate young cells (48 h in YM agar) in Ribitol agar.
2. Incubate plates for 1 week at 17°C.
3. Compare with reference strain or with Fig. 2.

3.2.5. Astaxanthin Biosynthesis

Extraction, detection, and quantification of astaxanthin are described below (see Subheading 3.4).

3.3. Molecular Identification of Wild *X. dendrorhous* Isolates

As described above, *X. dendrorhous* has a unique combination of phenotypic characteristics that in some cases may be sufficient for an appropriate identification. However, nowadays, accurate identification requires the use of molecular techniques. Molecular techniques, such as DNA sequencing, allow phylogenetic analysis using additional sequences from public gene databases (e.g., GenBank). Useful regions for *X. dendrorhous* identification through DNA sequencing are the gene-encoding D1/D2 domains of the large ribosomal subunit (26S) and the region comprising the ITS1 and 2, and the 5.8S ribosomal subunit (hereinafter D1/D2 and ITS regions, respectively). The D1/D2 region is highly conserved in *X. dendrorhous*, and it is a good choice for accurate identification. The ITS region not only allows correct species assignation but also, given to a high intraspecific variability in *X. dendrorhous*, may provide information for distinguishing natural populations (8).

1. Prepare a fresh culture of the *X. dendrorhous* strain on YM agar.
2. Extract the genomic DNA using appropriate commercial kits or alternative procedures (see Note 5).
3. Amplify by polymerase chain reaction (PCR) a specific region of rRNA genes or using primers ITS5 and LR6. Use the following PCR program: (1) Initial denaturing step at 95°C for 5 min. (2) 40 cycles of 45 s at 93°C, 60 s at 50°C, and 60 s at 72°C. (3) A final extension step of 6 min at 72°C. Expect a fragment size of approximately 1,400 bp.
4. Purify the corresponding PCR product using an appropriate extraction kit, and sequence it using the pair of primers NL1/NL4 for D1/D2 region (ca. 660 bp) and/or ITS1/ITS4 (ca. 715 bp) for ITS region (see Note 6).

3.4. Extraction, Detection, and Quantification of Astaxanthin

X. dendrorhous strains typically produce astaxanthin as their major carotenoid pigment (approximately 80% of total carotenoids); consequently, this yeast possesses significant biotechnological relevance as a natural source of astaxanthin pigment for aquaculture feed. Other minor carotenoids produced by *X. dendrorhous* are β-carotene, echinenone, 3-hydroxyechinenone, phoenicoxanthin, and canthaxanthin (15). This pool of pigments substantially differs from that of the most pigmented yeasts, such as those of the genus *Rhodotorula*. Astaxanthin is a xanthophyll and is highly polar; hence, in HPLC chromatograms it normally appears first (Fig. 3a). *X. dendrorhous* extracts UV–Vis spectra has a typical broad spectrum band with no evident absorption peaks and a maximum at 477 nm (Fig. 3b).

1. Harvest the cells of 20 mL of culture by centrifugation at 16,000 × *g*.
2. Wash twice with distilled water.
3. Suspend the pellet in 4.5 mL of distilled water and divide in four aliquots: two tubes with 1 mL each for carotenoid extraction, and two previously tared tubes with 1 mL each for dry weight.
4. For carotenoid extraction, add 1.5 mL of glass beads (425–600 μm Ø) and incubate on ice for 2 min.
5. Mix in vortex for 2 min.
6. Add 2 mL of acetone and incubate on ice for 2 min.
7. Mix in vortex for 2 more minutes.
8. Centrifuge at 1,500 × *g* and collect the supernatant in clean tubes. Store at −20°C.
9. Repeat the process until a white pellet is obtained.

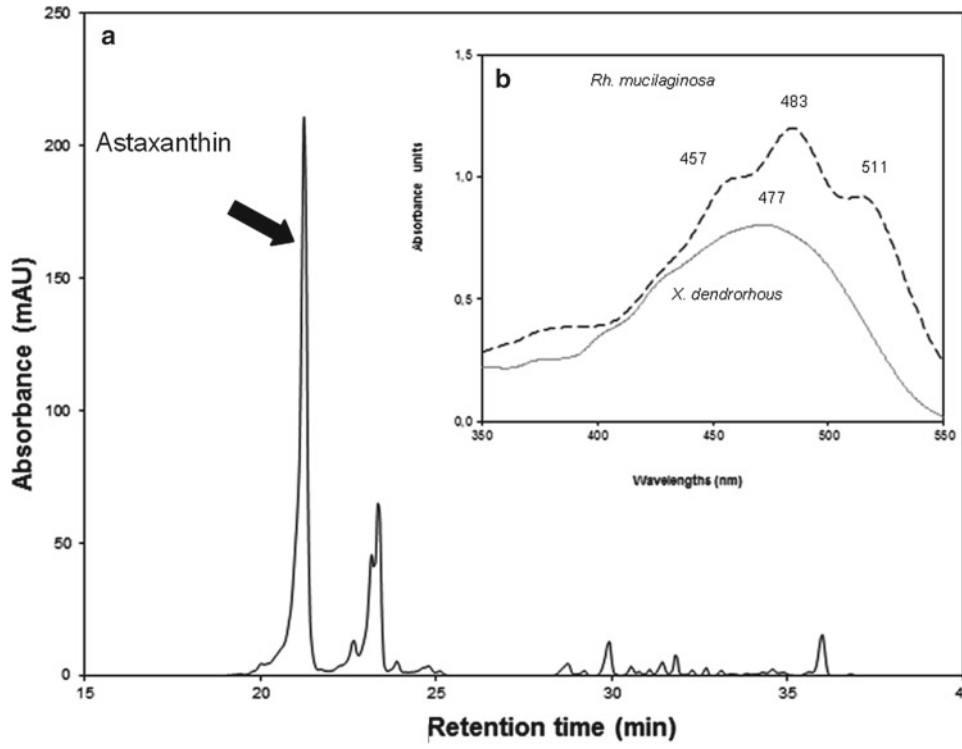

Fig. 3. Spectrophotometric and HPLC analysis of *Xanthophyllomyces dendrorhous* extracts. (**a**) Typical HPLC chromatogram of *X. dendrorhous*. (**b**) Comparison of carotenoid extracts UV–visible spectra with *Rhodotorula mucilaginosa* (ubiquitous pigmented yeast). *AU* absorbance units.

10. Add 2 mL of petroleum ether (35–60°C) at 5°C to the tube containing the supernatants and mix in vortex for 2 min.
11. Centrifuge the tubes at 10,000 × *g* for 10 min at 5°C and collect the supernatant, which contains the pigments, in a new tube.
12. Evaporate the petroleum ether under a continuous nitrogen flux.
13. Add 1 mL of *n*-hexane or petroleum ether for spectrophotometric quantification.
14. Measure the absorbance of the sample at 474 nm.
15. Estimate total carotenoid concentration using the following simplified formula.

$$\text{Carotenoids}_{t}(\mu g/g) = \frac{A_{474} \times 1 \times 10^{4}(\mu g/mL)}{2{,}100 \times \text{dw}(g/mL)}.$$

A_{474}: average absorbance (474 nm) of the two tubes (see step 11), dw: average dry weight of the two tubes (see step 2), and 2,100: extinction coefficient of astaxanthin.

16. For HPLC detection of astaxanthin, suspend extracts in 100 µL acetone.
17. Analyze the carotenoid content using a C18 column, an isocratic mobile phase consisting of 85% acetonitrile/10% methanol/5% isopropanol, and a flow rate of 1 mL/min.
18. Monitor the eluted fractions with a photodiode array detector, scanning from 270 to 600 nm every 2 s.
19. Identify carotenoids by their retention times and by comparison of the spectral features with those of pure compounds or with those of a reference strain.

3.5. Preservation of X. dendrorhous Wild Strains

Long-term preservation of fungal strains is essential for their in-depth study; however, both the viability and the stability of living cells should be ensured during the preservation period. Wild isolates of *X. dendrorhous* normally do not survive long on regular culture media and need to be subjected to cryopreservation for prolonged preservation. Different methods have been reported for yeast preservation (16, 17); although, the method described in this subheading has proven to be effective for preserving *X. dendrorhous* wild strains (see Note 7).

1. Prepare a fresh culture of *X. dendrorhous* strain on a small YM agar plate.
2. Spread 1 mL of a sterile solution of 20% v/v glycerol over the colony.
3. Add 10–15 glass beads (2–3 mm diameter) onto the plate.
4. Use a sterile small spoon to mix beads, glycerol, and yeast biomass, and transfer yeast-soaked beads into a sterile cryotube.
5. Store tubes at −80°C (see Note 8).
6. To obtain a fresh culture, remove one of the beads using a sterile pair of tweezers; rub bead on YM agar plate and incubate at 17°C.

4. Notes

1. This species normally constitutively produces detectable levels of MYCs, though photostimulation is recommended. Any illuminating device with a significant light intensity will stimulate Mycosporine synthesis in *X. dendrorhous*. Temperature control is important when incubating under light due to the heat emitted by the lamps.
2. An additional 2–3 days at 5°C helps to intensify colony colors, and thus helps in the detection of *X. dendrorhous*-like colonies, which are orange or salmon-orange.

3. *X. dendrorhous* colonies turn to a characteristic intense blue to violet color when flooded with diluted lugol.
4. A positive result is obtained when gas is accumulated inside the inverted Durham tube.
5. An alternative DNA extraction procedure is described by Libkind et al. (18). Briefly, two loopfuls of YM agar grown cultures are suspended in Eppendorf tubes containing 500 µL of lysing buffer and a volume of 200 µL of 425–600 µm of glass beads. After vortexing for 3 min, tubes are incubated for 1 h at 65°C. After vortexing again for 3 min, the suspensions are centrifuged for 15 min at 4°C and 20,000 × *g*. Finally, the collected supernatant is diluted 1:750, and 5 µL are used directly for PCR studies.
6. Type strain of *X. dendrorhous* is CBS 7918 and the rDNA sequences for comparison are AF075496 (D1/D2) and AF139628 (ITS).
7. An alternative preservation method for *X. dendrorhous* mutants, based on dehydrated gelatin drops, was described by Baeza et al. (19).
8. In our experience, survival is improved if tubes are initially stored at −20°C (2–3 days) and then transferred to −80°C.

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