Chapter 18

Isolation and Selection of New Astaxanthin-Producing Strains of *Phaffia rhodozyma*

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Abstract

Astaxanthin is a xanthophyll pigment of high economic value for its use as a feeding component in aquaculture. *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) is a basidiomycetous fungi able to synthesize astaxanthin as its major carotenoid, the only known yeast species bearing the capability to produce this type of carotenoid and the only tremellomycetes with biotechnological application. 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 *P. rhodozyma* 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 *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) 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 *P. rhodozyma*, 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-leafed trees in different mountainous regions in the northern hemisphere, such as Japan and Canada. More recently, isolations of this species have been carried out in Italy [6], Germany [7], Argentina [8], the USA [9], and Antarctica [10].

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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, and all Patagonian strains of *P. rhodozyma* bear genetic differences with collection *P. rhodozyma* strains. Even more recently, a study of association of *Phaffia* with *Nothofagus-Cyttaria* in Australasia [11], the other region of the world where *Nothofagus* are endemic, revealed an ever higher *Phaffia* diversity, including two endemic and markedly divergent lineages which represent putative new species, both with the capability to produce astaxanthin and mycosporine-glutaminol-glucoside (MGG). The genetic diversity that was described in *P. rhodozyma* correlates with the observed association with the tree genera (*Betula*, *Cornus*, and *Nothofagus*), rather than with geography, and constitutes seven different lineages. Later, an even more divergent strain was found in soil samples [12].

Thus, it becomes clear that the natural geographic distribution of *Phaffia* is wider than expected and that its habitat is not restricted to slime fluxes but rather includes sugar-rich fungal stromata, leaves, soil, and freshwater. Furthermore, the genetic variability of this astaxanthin-producing yeast is also more diverse than what was previously thought, and recent genetic evidence indicates that there is more than one species in the genus [11, 12]. A draft genome sequence of the type strain of *P. rhodozyma* became recently available, and comparison with that of the Patagonian population suggested the latter deserves to be assigned to a distinct variety [13]. In this work, the genetic basis of several photoprotective and antioxidant strategies is described, indicating that *P. rhodozyma* is one of the fungi most well equipped to cope with environmental oxidative stress, a factor that has probably contributed to shaping its genome. The biotechnological potential of the Patagonian isolates has only been preliminary assessed [14].

In the present work, we describe the strategies currently used in our laboratory for the effective isolation of *Phaffia* spp. from environmental samples, the rapid and 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 P. rhodozyma from Environmental Samples

1. YM agar: 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L dextrose, 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, 20 g/L agar. Adjust pH 4.5–5.0 and supplement with 200 mg/L chloramphenicol.
3. YNB + Tre agar (selective isolation medium): 6.7 g/L YNB (yeast nitrogen base, DIFCO), 5 g/L trehalose, 20 g/L agar, and supplement with 200 mg/L chloramphenicol (*see* **Note 1**).
4. 0.9% NaCl.
5. Filter membranes (0.45 μm) (Millipore Corporation, Bedford, Massachusetts, USA).
6. Sterile filtering apparatus.

2.2 Identification of Wild P. rhodozyma 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 2**).
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 P. rhodozyma 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. ITS3 forward primer 5′-GCA TCG ATG AAG AAC GCA GC-3′.

8. ITS4 reverse primer 5′-TCC TCC GCT TAT TGA TAT GC-3′.
9. PhR reverse primer 5′- GAC TTG TAC ACA GGC CGG CA-3′.
10. PCR reaction mix kit.
11. Thermocycler.
12. Genomic DNA extraction kit.
13. Lysing buffer: 50 mM Tris–HCl, 250 mM NaCl, 50 mM EDTA, and 0.3% w/v SDS, pH 8.
14. PCR product purification kit.

2.4 Extraction, Detection, and Quantification of Astaxanthin

1. C18 HPLC column (5 μm, 250 by 4.6 mm).
2. Acetonitrile/methanol/isopropanol: 40 mL acetonitrile, 50 mL methanol, 10 mL isopropanol.
3. Astaxanthin standard (Sigma, St. Louis, MO, USA).
4. Carotenoid extract from a reference *P. rhodozyma* collection strain (e.g., CBS 7918^{T}) [15].

2.5 Preservation of P. rhodozyma 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 P. rhodozyma from Natural Environments

To date, *P. rhodozyma* 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 *P. rhodozyma*, have an orange-characteristic color. Photos of fluxes colonized by *P. rhodozyma* 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 Southern Hemisphere. However, *P. rhodozyma* 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, 11, 16]. In most environmental samples, it has been observed that isolation frequency is very low; thus a partially

selective culture medium with trehalose as sole carbon source has been developed (here referred as YNB + Tre agar) [17]. This selective isolation medium quite effectively inhibits growth of most of the white co-occurring yeasts which typically appear with higher abundance and growth rates than *P. rhodozyma* (*see* **Note 3**). 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 P. rhodozyma 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 P. rhodozyma from Stromata Samples

1. Collect aseptically stromata of *Cyttaria hariotii*, an ascomycetous fungus parasite of *Nothofagus 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 P. rhodozyma from Exudate Samples Leaves Samples

1. Collect aseptically whole leaves (as fresh as possible).
2. Place leaves (1–15 depending on size) in a 10–15 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 P. rhodozyma 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 P. rhodozyma 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 sterile glass wool.

3. Do dilution series in fresh medium.
4. Plate out on agar plates.

Recommended temperature range for plate incubation is 15–20 °C, with a 17 °C optimum. Normally, 5 days of incubation are enough for colony emergence (*see* **Note 4**). Transfer orange colonies to YM or YPD fresh medium and purify.

3.2 Identification of Wild *P. rhodozyma* Using Phenotypic Characteristics

P. rhodozyma 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 *P. rhodozyma* isolates to the minimum. The distinctive combination of phenotypic traits includes the ability to produce amyloid compounds, glucose fermentation capabilities [18], mycosporine-glutaminol-glucoside (MGG) production (Fig. 1) [19], development of unique sexual structures (Fig. 2) [15], and synthesis of astaxanthin as a main carotenoid pigment [20]. Any one of these features can be tested to eliminate non *P. rhodozyma* isolates, and the combination of all of these features will provide an accurate identification. In particular, unlike any other yeast species, *P. rhodozyma* simultaneously produces MGG and astaxanthin, both of which can be extracted in a single step [17]. The characteristic spectrum of extracts consists of a narrow peak, with a maximum absorbance at 309–310 nm, and a broad spectral band with a maximum at 477 nm, corresponding to MGG and astaxanthin, respectively (Figs. 1 and 3). This method is particularly useful for rapid

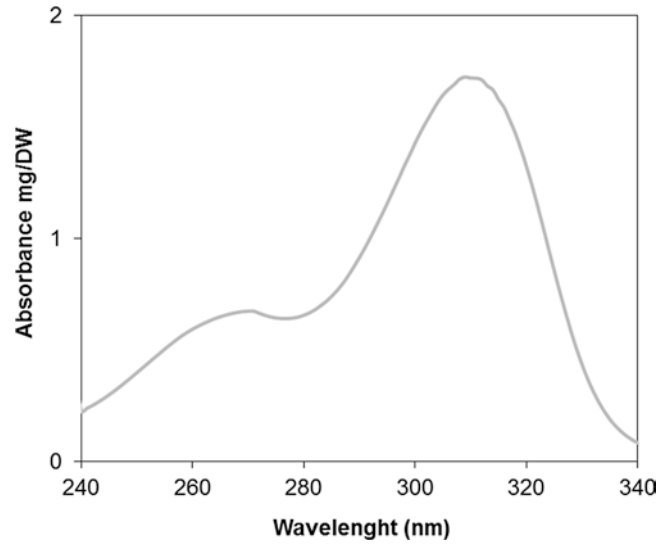

Fig. 1 UV-visible spectrum of mycosporine-glutaminol-glucoside in *P. rhodozyma* extract. *DW* dry weight

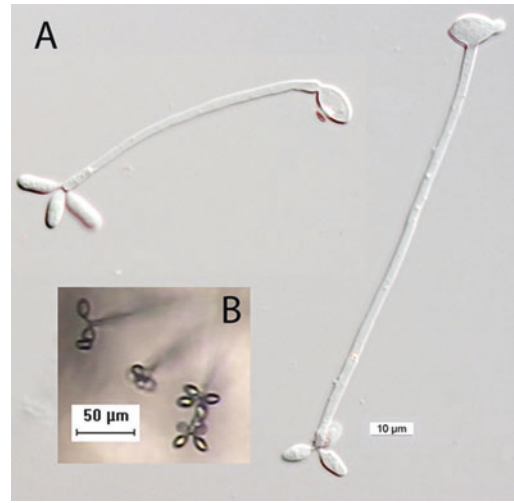

Fig. 2 Sexual structures of *P. rhodozyma*. (**a**) Daughter cell-mother cell conjugation (pedogamy) and holobasidium with terminal basidiospores. (**b**) Terminal basidiospores

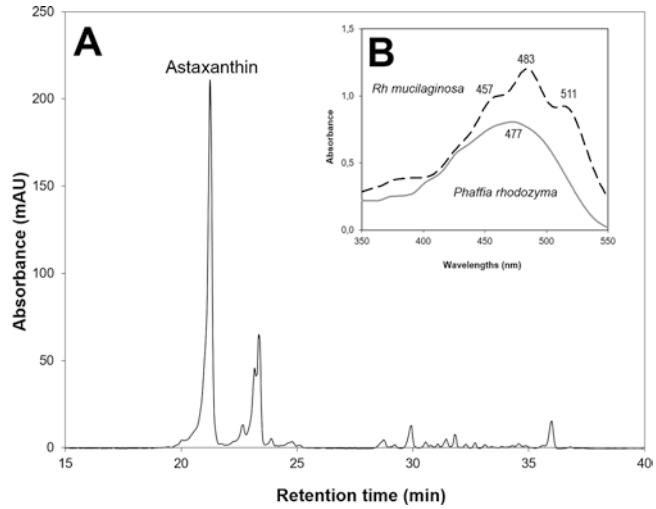

Fig. 3 Spectrophotometric and HPLC analysis of *P. rhodozyma* extracts. (**a**) Typical HPLC chromatogram of *P. rhodozyma*. (**b**) Comparison of carotenoid extracts UV-visible spectra with *Rhodotorula mucilaginosa* (ubiquitous pigmented yeast)**.** *AU* absorbance units

identification to distinguish *P. rhodozyma* from other pigmented yeasts that share its habitat, e.g., *Cystofilobasidium* cannot release MGG nor carotenoids into extracts, while yeasts able to produce MGG have a different carotenoid spectrum which in most cases are absent (*A. pullulans* and *Rhodotorula* spp.) or consisted of three peaks around 455, 484, and 520 nm (*Dioszegia* spp.).

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 5**).

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 6**).

3.2.3 Mycosporine Production

1. Grow yeast isolates on YM agar plates under illumination conditions for 3 days at 20 °C (*see* **Note 2**).
2. Harvest biomass and transfer to Eppendorf tube containing 1 mL of 80% (v/v) ethanol.
3. Agitate vigorously with a vortex mixer and incubate in 85 °C bath for 2 h.
4. Clear extracts by centrifugation at 10,000 × *g* for 10 min.
5. Obtain full spectrum within range 190–700 nm in an UV-visible spectrophotometer using quartz cuvettes.
6. Compare with reference strain or with Fig. 1.

This method simultaneously releases astaxanthin together with MGG, allowing a rapid identification screen for pigmented yeasts, as it was mentioned above.

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

For astaxanthin biosynthesis, *see* Subheading 3.4.

3.3 Molecular Identification of Wild *P. rhodozyma* Isolates

As described above, *P. rhodozyma* 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.

DNA sequencing allows phylogenetic analysis using additional sequences from public gene databases (e.g., GenBank). Useful regions for *P. rhodozyma* identification through DNA sequencing are the gene encoding the D1/D2 domains of the large ribosomal subunit (26S) and the region comprising the Internal Transcribed Spacers (ITS) 1 and 2, and the 5.8S ribosomal subunit (hereinafter D1/D2 and ITS regions, respectively). The D1/D2 region is highly conserved in *P. rhodozyma*, 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 *P. rhodozyma*, may provide information for distinguishing natural populations [8].

On the other hand, a quick and accurate PCR-based detection method was developed to detect isolates belonging to all known lineages of the genus *Phaffia* [21]. For this purpose, a primer of 20 nucleotides (called PhR) was designed to be used in combination with universal primers ITS3 and NL4 in a multiplex amplification. The reverse PhR primer was designed to anneal in the conserved D1/D2 domains of the 26S rRNA gene and to generate a 643 bp amplicon when it is used together with the universal forward primer ITS3. The universal reverse primer NL4 was included for amplification control since it will produce a single fragment around 1155 bp in any eukaryotic microorganism. The use of three primers in a multiplex reaction has the advantage of still obtaining an amplicon in a negative sample; therefore, the results are unambiguous and cannot be attributable to a failure in the amplification reaction.

1. Prepare a fresh culture of the *P. rhodozyma* strain on YM agar.
2. Extract the genomic DNA using appropriate commercial kits or alternative procedures (*see* **Note 7**).
3. Polymerase chain reaction (PCR).
   (a) PCR-based detection method: Amplify using primers ITS3, PhR, and NL4. Use the following PCR program: (1) initial denaturing step at 95 °C for 5 min. (2) Thirty-five cycles of 30 s at 95 °C, 60 s at 55 °C, and 60 s at 72 °C. (3) A final extension step of 7 min at 72 °C (*see* **Note 8**).
   (b) DNA sequencing: Amplify 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) Forty 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 1400 bp.
4. For DNA sequencing, 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 9**).

3.4 Extraction, Detection, and Quantification of Astaxanthin

P. rhodozyma 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 *P. rhodozyma* are β-carotene, echinenone, 3-hydroxyechinenone, phoenicoxanthin, and canthaxanthin [20]. This pool of pigments substantially differs

from that of 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). *P. rhodozyma* 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 min.
8. Centrifuge at 1500 × *g* and collect the supernatant in clean tubes. Store at −20 °C.
9. Repeat the process until a white pellet is obtained.
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.

$$\text{Carotenoids}_t\left(\mu g / g\right) = \frac{A_{474} \times 1 \times 10^4 \left(\mu g/mL\right)}{2100 \times dw\left(g/mL\right)}$$

A_{474}: average absorbance (474 nm) of the two tubes (*see* **step 11**).
dw: average dry weight of the two tubes (*see* **step 2**).
2100: 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 *P. rhodozyma* 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 *P. rhodozyma* 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 [22, 23], although the method described in this Subheading has proven to be effective for preserving *P. rhodozyma* wild strains (*see* **Note 10**).

1. Prepare a fresh culture of *P. rhodozyma* 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 11**).
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. Colony size on YNB + Tre agar is about 50% smaller than conventional medium. This problem can be solved by increasing trehalose concentration to 8–10 g/L, which increase the size of *P. rhodozyma* colonies obtained to comparable levels to that of the conventional medium, thus facilitating colony detection and picking.
2. 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 *P. rhodozyma*. Temperature control is important when incubating under light due to the heat emitted by the lamps.

3. The fact that samples positive for *P. rhodozyma* in YNB + Tre agar medium are usually negative in conventional medium (YM agar) and vice versa, it is suggested that the best strategy to increase the recovery rate of *P. rhodozyma* from environmental samples is the use of both media.
4. An additional 2–3 days at 5 °C helps to intensify colony colors and thus helps detection *P. rhodozyma*-like colonies, which are orange or salmon orange.
5. *P. rhodozyma* colonies turn to a characteristic intense blue to violet color when flooded with diluted lugol.
6. A positive result is obtained when gas is accumulated inside the inverted Durham tube.
7. An alternative DNA extraction procedure is described by Libkind et al. [24]. 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.
8. Expect a fragment size of 643 bp if the sample belong to the genus *Phaffia* with a faint band of approximately 1155 bp. Negative samples show a single 1155 bp band. This method proved efficient for all lineages reported by David-Palma et al. [11], though it did not work for the divergent strain TSN-67 reported by Yurkov et al. [12].
9. Type strain of *P. rhodozyma* is CBS 7918, and the rDNA sequences for comparison are AF075496 (D1/D2) and AF139628 (ITS).
10. An alternative preservation method for *P. rhodozyma* mutants, based on dehydrated gelatin drops, was described by Baeza et al. [25].
11. 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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