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Favored isolation and rapid identification of the astaxanthin-producing yeast *Xanthophyllomyces dendrorhous*(*Phaffia rhodozyma*) from environmental samples

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Xanthophyllomyces dendrorhous (*Phaffia rhodozyma*) yeasts are biotechnologically exploited as a natural source of astaxanthin for aquaculture. Based on results of recent studies, it has become clear that this species possesses a greater genetic variability generating the necessity to uncover it and assess its potential for the astaxanthin industry. However, difficulties for the isolation of the *X. dendrorhous* hinder extensive environmental surveys which need to be carried out to better understand the habitat, distribution and genetic diversity of this species. We extensively searched for distinctive physiological traits of *X. dendrorhours* by testing phenotypic properties simultaneously with a panel of common sympatric fungi. As a result we obtained a new and innovative strategy for improving *X. dendrorhous* recovery rate and identification from environmental samples. This strategy involved the use of trehalose-based media, and a rapid *X. dendrorhous* identification method based on the simultaneous spectrophotometric detection of astaxanthin and UV-absorbing compounds (mycosporines). The proposed procedures proved effective in field trials conducted in natural environments of Patagonia (Argentina) and thus represent an important tool for the discovery of new astaxanthin-producing strains of *X. dendrorhous* useful for the aquaculture industry.

Abbreviations: MGG – mycosporine-glutaminol-glucoside; MYCs – mycosporines; Tre – trehalose; CRUB – Centro Regional Universitario Bariloche yeast Collection, Universidad Nacional del Comahue, Argentina; UCD – Phaff Yeast Culture Collection, University of California at Davis, USA; CBS – Centraalbureau voor Schimmelcultures, Netherlands; PYCC – Portuguese Yeast Culture Collection, Universidade Nova de Lisboa, Portugal; YNB – Yeast Nitrogen Base.

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Introduction

Xanthophyllomyces dendrorhous (sexual stage of *Phaffia rhodozyma*) is a basidiomycetous yeast that develops pink to red colonies due to the accumulation of the carotenoid pigment astaxanthin, an economically important pigment that is absent in other yeasts [1]. Astaxanthin is the most expensive aquaculture feed component and as a result *X. dendrorhous* is biotechnologically produced and commercially sold as a natural source of this pigment [2, 3].

Habitats and distribution of *X. dendrorhous* are broader than originally suspected. The first isolations of *X. dendrorhous*, which have served for most of the studies involving this species, were obtained in the 1960s by Phaff and collaborators [4] from slime exudates of various broad-leafed trees in Japan and Canada. More recent isolations from slime fluxes were carried out in Italy [5], Germany [6], and USA [7]. Finally, distribution of *X. dendrorhous* was shown to extend into the Southern hemisphere, where it grows associated to the sugary stromata of *Cyttaria hariotii* (Argentina) [8] or leaves (Chile) [9].

Newer *X. dendrorhous* isolations have shown that this species also presents a broad genetic diversity. Based on

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rDNA ITS sequence analysis, Argentine strains form a genetically uniform and distinct population that is different from the one that includes type strains [8, 10]. Furthermore, the Chilean strain has marked ITS and 26S rDNA-based phylogenetic differences with the other known populations, and might even represent a novel species [9], though additional strains would be necessary for an appropriate description.

Habitat, distribution and genetic diversity of *X. dendrorhous* may provide valuable insight into the biotechnological and evolutionary aspects of this species. However, difficulties for the isolation of *X. dendrorhous* hinder extensive surveys which need to be carried out for this purpose, especially in the fairly unexplored regions of the southern hemisphere such as Patagonia. Specifically, isolation frequency in environmental samples from Patagonia is very low. Furthermore, *X. dendrorhous* may be readily confused with other pigmented yeasts that share its habitat. For example, *Cystofilobasidium* yeasts are frequently isolated simultaneously with *X. dendrorhous* in natural substrates [11]. Considering that *Cystofilobasidium* yeasts grow in orange colonies, produce amyloid compounds, and may sporadically weakly ferment glucose, colonies of this yeast may initially be misclassified as *X. dendrorhous*-like. However, it has recently been discovered that *X. dendrorhous* produces the UV absorbing mycosporine, mycosporine-glutaminol glucoside (MGG) [12], which is not present in *Cystofilobasidium* species.

The objectives of the present study were to develop a favorable medium for the isolation of *X. dendrorhous*, and to provide a rapid detection method for screening the pigmented colonies that are recovered during environmental surveys. For both objectives, special emphasis was placed on trials using environmental samples.

Materials and methods

Design of selective isolation media

Based on a preliminary survey of 35 different C sources for the differential isolation of *X. dendrorhous* with respect to other co-occurring yeasts, trehalose, melezitose, and arbutin were selected for the design of a favorable isolation medium. Strains used for this study were: 26 native Patagonian *X. dendrorhous* strains, reference *X. dendrorhous* strains (CBS: 7918^T, 5908, 6938, 7919, PYCC 4172, and CBS 5905^T), and 19 non-*X. dendrorhous* yeast strains (Table 1) corresponding to pigmented and

Table 1. Growth of pure cultures of *X. dendrorhous*, *P. rhodozyma*, and non-*X. dendrorhous* strains on test media (6 d growth).

	C source		
Species	**Trehalose**	**Melezitose**	**Arbutin**
X. dendrorhous			
Patagonian strains[a]	+	+	+
Collection strains[b]	+	+	+
Non-*X. dendrorhous*[c]			
Pigmented			
Aureobasidium pullulans	+	+	+
Sporidiobolus longiusculus	−	+	−
Rhodotorula mucilaginosa	+	+	+
Rhodotorula mucilaginosa	+	+	+
Rhodotorula colostrii	+	+	+
Cystofilobasidium infirmominiatum	−	+	+
Cystofilobasidium capitatum	+	+	+
Cystofilobasidium capitatum	+	−	+
Cystofilobasidium macerans	+w	−	+
Non-pigmented			
Mrakia frigida	+	+	+
Candida sp.	+	+	−
Candida maritima	+	+	+
Saccharomyces uvarum	−	+	−
Saccharomyces eubayanus	−	+	−
Saccharomyces cerevisiae	−	−	−
Kloeckera sp. 1	−	−	+
Kloeckera sp. 2	−	−	+
Zigosaccharomyces cidri	+	+w	+
Pichia delftensis	−	+w	−

[a]26 Patagonian strains (see Materials and methods section for details).
[b]5 collection strains, including *P. rhodozyma* (see Materials and Methods section for details).
[c]Native strains belonging to the Centro Regional Universitario Bariloche (CRUB) collection.

non-pigmented yeasts that co-inhabit *Cyttaria* sp. stromata with *X. dendrorhous*.

Solid YNB (Yeast Nitrogen Base, DIFCO) 0.67% w/v, supplemented with trehalose, melezitose, or arbutin (0.5% w/v), was spot inoculated with pure cultures of the corresponding yeast according to Yarrow [13]. Plates were duplicated, and negative (no C source) and positive controls (0.5% w/v glucose) were included. Colony growth was checked after 3, 6, 8, 14 and 21 d.

The tolerance to different glucose concentrations was tested using 5 representative strains of *X. dendrorhous* (CRUB 853, 1149, and 1614; CBS 7918^T; and UCD 67.203), *Phaffia rhodozyma* (CBS 5905^T), two species of the genus *Rhodotorula* (*R. mucilaginosa* strains CRUB 243 and 299; and *R. colostrii* CRUB 775), and 3 species of thegenus *Cystofilobasidium* (*C. infirmominiatum* strain CRUB 305, *C. capitatum* strain CRUB 796, and *C. macerans* strain CRUB 1174). Plates with solid YNB (0.67% w/v) supplemented with glucose at concentrations of 20, 30, 40 y 50% [13] were spot inoculated with pure cultures of the corresponding yeasts as previously described. Plates were triplicated, and negative (no glucose) and positive (0.5% glucose) controls were included. Growth was registered after 3, 6, 8, and 14 d.

Tolerance to different ethanol concentrations was investigated using the same *X. dendrorhous* strains as the glucose concentration tests were used, plus the non-pigmented yeast *Zigosaccharomyces cidri* (strain CRUB 1569). Plates with solid YNB (0.67% w/v) supplemented with glucose at a concentration of 0.5%, and concentrations of ethanol of 2, 4, 6–9, and 10% [13] were spot inoculated, as previously described, with pure cultures of the corresponding yeast strains. Plates were duplicated, and negative (no ethanol and no glucose) and positive (no ethanol) controls were included. Growth was registered after 3, 6, 8, and 14 d.

Rapid identification of *X. dendrorhous* based on its ability to simultaneously produce mycosporines (MYCs) and astaxanthin

A set of 42 strains of 20 different yeast and yeast-like species, together with 20 collection (native and reference) *X. dendrorhous* strains, and suspected *X. dendrorhous* yeast strains (see below) were transferred to MMS (10 g liter^{-1} glucose, 2 g liter^{-1} $(NH_4) \cdot 2\ SO_4$, 2.5 g liter^{-1} $KH_2(PO_4)$, 0.5 g liter^{-1} $MgSO_4 \cdot 7\ H_2O$, 1 g liter^{-1}, 1.5 g liter^{-1} Agar-Agar) and incubated at 20 °C for 72 h under white light (1.93 W s^{-1} m^{-2}), in an environmental test chamber (Semedic I-500PF). The species selected represent the main 4 phylogenetic groups containing most known red yeasts [14] and other species also producing conspicuous amounts of carotenoid pigments.

Biomass of each strain (two loopfulls) was suspended in 1 ml ethanol 80% (v/v) in Eppendorf tubes, vigorously agitated with a vortex mixer, and incubated in an 85 °C bath for 2 h. Tubes were then centrifuged at 10,000 rpm and the supernatant was spectrophotometrically measured in the range of 190–700 nm. Absorbance profiles were compared to that of the *X. dendrorhous* type strain. The production of mycosporines was registered as the presence of a peak at 310 nm, and the maximum peak/s from the extracted carotenoids was also registered in the 450–600 nm range. The presence of astaxanthin and mycosporines (in particular mycosporine-glutaminol-glucoside) in collection strains and patagonian strains had been previously confirmed using HPLC [3, 10, 12], and these strains served as reference for this study.

Field assays using selective test media and rapid identification screen for pigmented yeasts

Sampling was carried out in Patagonian National Parks between December 2009 and January 2010 (spring-summer). Samples of *Cyttaria hariotii* growing off *Nothofagus sp*. and/or leaves of these trees were collected aseptically and stored in refrigerated sterile flasks till processing upon arrival at the laboratory.

Isolation of *X. dendrorhous* from *Cyttaria* spp. was carried out as described by Libkind *et al*. [8], with minor modifications. Ascostromata were placed in bags with sterile distilled water (1:1 w/v), and crushed manually inside the bags. Leaves (approximately 10–15) were transferred to sterile 25 or 50 ml tubes (depending on the size of the leaves), covered with sterile distilled water (10–50 ml) and shaken (INNOVA 4000) for 24 h. Aliquots of 100 µl of the *Cyttaria* sp. or leaf extracts were inoculated on test media, i.e. solid YNB supplemented with 0.5% w/v trehalose (YNB + tre) or control media (YM agar: 3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone; 10 g/l dextrose; 20 g/l agar). For the case of YNB + tre, extracts were not diluted, whereas the control media was diluted accordingly to avoid overcrowded plates. Culture media were supplemented with 200 mg/l chloramphenicol. Incubation temperature was 20 °C. Orange to pink-salmon colonies were transferred to fresh YM agar plates and kept at 4 °C.

Suspected *X. dendrorhous* strains were subjected to the rapid detection method, and five selected strains that tested positive were confirmed by sequencing the region comprising the internal transcribed spacers (ITS)1 and 2 and the 5.8S rRNA gene as previously described [8].

Results

All *X. dendrorhous* strains had positive growth on trehalose, melezitose and arbutin media (Table 1).

Table 2. Growth of pure cultures of *X. dendrorhous* and non-*X. dendrorhous* strains on media with different concentrations of glucose or ethanol (6 d growth).

	Concentration (% w/v)								
	Glucose				Ethanol				
Species	20	30	40	50	2	4	6	10	20
X. dendrorhous									
Patagonian strains[a]	+	+	+	−/w[c]	+	+	−	−	−
Collection strains[b]	+	+	+	−	+	+	−	−	−
Non-*X. dendrorhous*[d]									
Pigmented									
Aureobasidium pullulans	Nd				+	+	−	−	−
Rhodotorula mucilaginosa	−	−	−	−	+	+	−	−	−
Rhodotorula mucilaginosa	−	−	−	−	+	+	+	−	−
Rhodotorula colostrii	−	−	−	−	+	+	+	−	−
Cystofilobasidium infirmominiatum	+	+	+	−	+	+	+	−	−
Cystofilobasidium capitatum[e]	+	+	+	−	+	+	+	−	−
Cystofilobasidium macerans	+	+	+	−	+	+	−	−	−
Non-pigmented									
Mrakia frigida	Nd				+	+	+	−	−
Candida sp.	Nd				+	+	+	−	−
Candida maritima	Nd				+	+	+	−	−
Zigosaccharomyces cidri	Nd				+	+	+	+	−

[a]CRUB strains 853, 1149, 1614.
[b]CBS 7918[T],UCD 67.203, and CBS 5905[T].
[c]Negative or weak growth.
[d]Native strains belonging to the Centro Regional Universitario Bariloche (CRUB) culture collection.
[e]For ethanol, two strains were tested, nd = not determined.

Colonies grown on trehalose had more intense pigmentation than that of colonies grown on other C sources or conventional culture media. Regarding the 21 non-*X. dendrorhous* yeasts tested, we observed absence of growth for 8 species in trehalose, 5 in melezitose and 6 in arbutin. All *X. dendrorhous* strains grew well at glucose concentrations of 20, 30 and 40% w/v, while growth was inhibited or was very weak at 50% w/v glucose concentration (Table 2). *Rhodotorula* spp. (both species) was inhibited by glucose concentrations above 20% w/v, while *Cystofilobasidium* spp. behaved like *X. dendrorhous*, growing well at glucose concentrations between 20–40%. Trehalose based medium was selected for field experiments.

The spectrophotometric analysis of a large set of *X. dendrorhous* isolates showed that this species has a characteristic spectrum, shown in Fig. 1. It consists ofa straight 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. The same protocol was applied to a set of yeast species with carotenoid containing colonies which could be potentially confused with *X. dendrorhous* in the isolation plates (Table 3). Species of the genus *Cystofilobasidium* were rapidly discarded, given they did not release MGG nor carotenoids into extracts. A similar situation occurred for *Taphrina*, *Pseudozyma*, *Sporobolomyces*, *Sporidiobolus* and most *Rhodotorula* species. The yeasts that shared the ability to produce MGG with *X. dendrorhous* were successfully differentiated from this species by their carotenoid spectra which in most cases was absent (*A. pullulans* and *Rhodotorula* spp.), or consisted

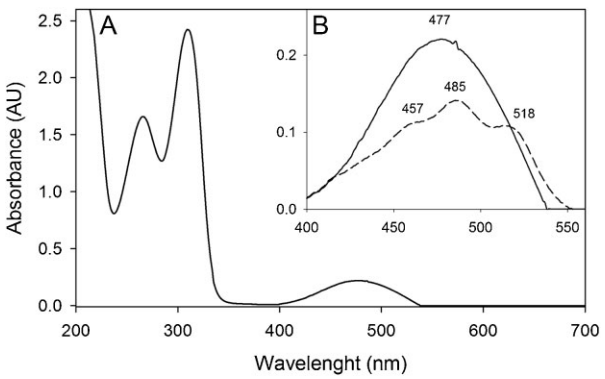

Figure 1. UV-visible spectra of *Xanthophyllomyces dendrorhous* ethanolic extracts (A); comparative visible spectrum of carotenoids from *X. dendrorhous* (solid line) and *Dioszegia* sp. (dashed line) (B).

Table 3. Presence of mycosporines and spectrophotometric profiles of pigments extracted in ethanol of *X. dendrorhous* and other pigmented yeasts.

Species	N° strains tested[a]	MYC	Absorption peaks (nm)
Xanthophyllomyces dendrorhous	20^T	+	477
Aureobasidium pullulans	1	+	–
Cystofilobasidium capitatum	2^T	–	–
Cystofilobasidium infirmominiatum	1^T	–	–
Cystofilobasidium lacus-mascardii	1^T	–	–
Dioszegia aurantiaca	1^T	+	463–489–524
Dioszegia cataronii	1^T	+	452–477–511
Dioszegia crocea	4^T	+	457–484–518
Dioszegia fristingensis	3	+	457–484–519
Dioszegia hungarica	2^T	+	457–484–518
Dioszegia sp.	3	+	457–484–518
Pseudozyma sp.	1	–	–
Rhodotorula araucariae	1^T	–	–
Rhodotorula glutinis	1^T	–	–
Rhodotorula lactosa	1^T	+	–
Rhodotorula minuta	2^T	+	–
Rhodotorula mucilaginosa	12	–	–
Rhodotorula pinicola	1^T	+	–
Sporidiobolus salmonicolor	1	–	–
Sporobolomyces ruberrimus	2^T	–	–
Taphrina carpini	1	–	–

[a]Except for type strains, all yeasts were native strains from Centro Regional Universitario Bariloche (CRUB) culture collection, identified by rRNA genes sequencing; ^{T} = type strain included.

of three peaks around 455, 484, and 520 nm (*Dioszegia* spp.).

Field experiments using both conventional yeast medium and the proposed trehalose based medium yielded a total of 82 *X. dendrorhous*-like strains, which were recovered from 90 environmental samples processed. These strains were subjected to the rapid screening method for identification of *X. dendrorhous*, and it was determined that 33 of them (corresponding to 20 samples) had a peak with maximum absorbance at 310 nm (MGG), and a typical xantophyll curve with maximum absorbance at 477 nm. For five representative strains, it was confirmed by ITS sequencing that they were, in fact, *X. dendrorhous*. New *X. dendrorhous* isolates had identical ITS sequences to those of previously studied Patagonian strains (i.e. DQ661028). The identity of the remaining *X. dendrorhous* strains was confirmed by sequencing a less conserved gene within ongoing investigations on the instraspecific variability of *X. dendrorhous* in Patagonia (data not shown). The *X. dendrorhous*-like isolates that did not pass the rapid screening were found to belong to the pigmented yeast genera *Cystofilobasidium*, *Rhodotorula* and *Dioszegia*.

Twenty environmental samples out of ninety yielded *X. dendrorhous* isolates, and almost half were possible due to the newly designed medium (Table 4). Only four samples had *X. dendrorhous* colonies simultaneously on both new and conventional media.

Table 4. Summary of *X. dendrorhous* field isolations.

Samples	Number
Total	90
Positive for *X. dendrorhous*[a]	20
Positive *only* on YM agar	8
Positive *only* on YNB + Tre agar	8
Positive simultaneously on *both* media	4

[a]As determined by results of rapid screening method for detection of *X. dendrorhous* and molecular identification (see Materials and methods section).

Discussion

The positive growth in trehalose, melezitose and arbutin observed for all Patagonian and collection *X. dendrorhous* strains was in agreement with previous reports [10, 15–17]. Palágyi *et al.* [17] also observed an enhanced pigmentation when this yeast was grown on Trehalose. In the present work, these characteristics were confirmed for a larger set of strains suggesting that are species specific. Trehalose quite effectively did not allow growth

of most of the white non-*X. dendrorhous* tested (i.e. *Saccharomyces* spp., *Kloeckera* spp. and *Pichia* sp.). This trait was important because these yeasts typically appear in environmental samples together with *X. dendrorhous*, and their abundance and growth rates are much higher [8, 12, 18].

None of the C-sources completely inhibited pigmented non-*X. dendrorhous* yeasts; thus, other potential selective characteristics were tested. Based on our findings, and in agreement with previous reports [16], media with 20–30% w/v glucose concentration would be adequate for growth of *X. dendrorhous*, but would only be effective for selecting against *Rhodotorula*. Tolerance to ethanol exhibited by *X. dendrorhous* ($\leq$4%) strains was similar or lower than that of non-*X. dendrorhous* (Table 2), thus, this characteristic did not prove adequate to include in a selective isolation strategy. The use of trehalose as the sole C source was selected for further experiments given it was quite effective against white yeasts, suggestingit might be helpful to increase environmental recovery rate of *X. dendrorhous*. However, given that mostpigmented yeasts were able to grow together with *X. dendrorhous* in trehalose the need of a rapid detection and differentiation method for the latter arose.

Unlike any other yeast species, *X. dendrorhous* simultaneously produces MGG and astaxanthin [12]. This combined feature was used to design the rapid identification method of *X. dendrorhous* in environmental samples. Previous exposure of the colonies to light induces MGG synthesis [12] and stimulates carotenoid synthesis in *X. dendrorhous* [19]. The ethanolic extraction procedure proposed ensures MGG release, and the same occurs with the more hydrophilic carotenoids like astaxanthin typical of *X. dendrorhous*. Laboratory trials against a large set of known pigmented yeast species showed that the rapid detection method here reported is precise and effective for the detection of *X. dendrorhous*. It is advisable to perform complementary analyses using HPLC [20] and rDNA sequencing methods for the analysis of the actual astaxanthin content and for reaching a final identification, respectively.

We later tested the new *X. dendrorhous* isolation medium (YNB + Tre agar) simultaneously with conventional medium for a large set of environmental samples. The addition of the newly designed media to the isolation protocol duplicated the chances of obtaining *X. dendrorhous* isolates. The fact that samples positive for *X. dendrorhous* in one of the media were generally negative in the other, and vice-versa, is a further indication of the difficulties that isolating *X. dendrorhous* from environmental samples presents. The fact that the same amount of samples was positive for test and conventional media suggests that the designed isolation media alone is probably not the best choice, and combination of both media to increase *X. dendrorhous* recovery rate from environmental samples is the best strategy.

X. dendrorhous colonies in YM-isolation plates displayed a light orange to salmon pigmentation which was similar to that showed by *Rhodotorula*, *Cystofilobasidium* and even some *Cryptococcus* species. On the contrary,on trehalose based medium, *X. dendrorhous* developed, deep-orange pigmented colonies that considerably reduced false positive colony picking. The fact that substrate extracts did not need to be diluted to inoculate trehalose medium was also advantageous, as it reduced workload. However, colony size, which was about 50% smaller on test media than conventional media, was sometimes an inconvenience, given that it made the search for colonies more difficult. This problem was later solved by increasing trehalose concentration to 0.8–1% w/v., which increased the size of the *X. dendrorhous* colonies obtained to comparable levels to that of the conventional media. It was also found that the 5th day of incubation was the best moment for colony selection, given that longer times allowed filamentous fungi to grow excessively and shorter times did not allow *X. dendrorhous* to develop sufficiently.

Conclusions

The physiological comparison of collection and wild strains of *X. dendrorhous* against other sympatric species allowed us to select trehalose as a partially selective carbon source for culture medium design. This medium favored mainly carotenogenic yeasts, and further attempts to use additional factors for more selective culture conditions for *X. dendrorhous* such as high glucose and ethanol concentrations failed. Thus, a rapid and effective method for *X. dendrorhous* identification among red yeast isolates was developed based on the simultaneous extraction and detection of MGG and astaxanthin.

Both the designed culture medium and the *X. dendrorhous* identification method were tested on environmental samples from Patagonia (Argentina). For the field studies, the use of the trehalose-based medium nearly doubled the number of positive samples, and should be used in combination with conventional medium for optimum isolation frequency. The identification method was an extremely convenient and precise technique for screening for *X. dendrorhous* among red yeast isolates, also being faster and cheaper than current molecular techniques. Thus, we provide a new and

innovative strategy for a higher *X. dendrorhous* recovery rate from the environment, which is of importance considering that there is evidence that this species possesses a larger genetic variability than previously suspected and due to its biotechnological and industrial relevance as a natural source of astaxanthin for aquaculture.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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