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ORIGINAL PAPER

Characterization of a novel South American population of the astaxanthin producing yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*)

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Abstract A novel population of the biotechnologically important yeast *Xanthophyllomyces dendrorhous*, the sexual stage of *Phaffia rhodozyma*, has been recently isolated for the first time in the southern Hemisphere (Patagonia, Argentina). The aim of the present work was to phenotypically and genotypically characterize two representative strains of this new population, and assess such strains as a potential biotechnological source of astaxanthin, fatty acids and extracellular enzymes. Minor variations were found in physiological tests. PCR fingerprinting studies (MSP-PCR) showed the main differences between *X. dendrorhous* Patagonian and Type strains. Patagonian strains accumulated a xanthophyll-like pigment, which was identified as astaxanthin. These strains showed low fatty acids content (mainly polyunsaturated fatty acids) and, of a total of six extracellular enzymes tested, only produced amylase. Genetic differences between Patagonian and collection *X. dendrorhous* strains could be explained by geographic isolation and habitat specificity.

Keywords Astaxanthin · *Phaffia rhodozyma* · *Xanthophyllomyces dendrorhous* · Patagonia · Strain characterization

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Introduction

Xanthophyllomyces dendrorhous (asexual stage, *Phaffia rhodozyma*) was first isolated by Herman Phaff in the early 1960's [20]. This yeast has a set of unique characteristics, which are of special interest to many scientists. It is the only known yeast capable of synthesizing astaxanthin, an economically important pigment widely used in fish and poultry feed [11]. *X. dendrorhous* also vigorously ferments sugars; this characteristic combined with the ability to synthesize carotenoids pigments is exclusive to this particular yeast species. The sexual stage of *X. dendrorhous* is also unusual, as it involves a slender holobasidia with terminal basidiospores formed after pedogamy-type conjugation in polyol-rich media [10, 13]. *Phaffia rhodozyma* Type strain (CBS 5905^T) is the only known anamorphic strain of this species. This condition, in addition to other phenotypic [12] and genotypic [17, 19] characteristics was originally used to consider this strain as a separate species [6, 14]. However, based on DNA–DNA reassociation studies, it was later demonstrated that *P. rhodozyma* and *X. dendrorhous* are anamorphic and teleomorphic states of the same species [16].

Original isolates of *X. dendrorhous* were obtained in Japan and the west coast of North America [20]. During the following years additional isolates were collected in Finland, Russia, Italy and Germany [9, 10, 30, 32]. Because all these isolates were found on exudates of broad-leaved deciduous trees belonging to genera *Betula*, *Alnus* and *Fagus* [10, 20], it was generally considered that *X. dendrorhous* grew exclusively in such habitats. However, recently, in the southern hemisphere, isolates of *X. dendrorhous* were found growing on sugar-rich fruiting bodies (stromata) of the ascomycetous fungus *Cyttaria hariotii* [16]. *C. hariotii* is an endemic parasite of *Nothofagus* spp.

trees in Patagonia, Argentina. The rDNA ITS sequence analysis showed that the new Patagonian *X. dendrorhous* population was genetically different from known *X. dendrorhous* strains.

The aim of this work is to phenotypically and genetically characterize the new South American population of *X. dendrorhous*, and to assess the biotechnological potential of the studied strains as a source of astaxanthin, fatty acids and extracellular enzymes.

Materials and methods

Yeast strains and reference cultures

Two representative strains of the Patagonian *X. dendrorhous* population were selected: strain CRUB 853 isolated from stromata of *C. hariotii*, and strain CRUB 1149, found in water samples of Lake Ilón (S 71°56′, W 41°11′, Nahuel Huapi National Park, Patagonia, Argentina). Sample collection and isolation procedures have already been described by Libkind et al. [16]. Reference cultures CBS 5905^T (*Phaffia rhodozyma*) and CBS 7918^T (*Xanthophyllomyces dendrorhous*) were kindly provided by Dr. Sampaio (CREM, Portugal). All the strains were kept on potato dextrose agar (PDA) at 4°C, and under liquid nitrogen.

Phenotypic characterization and sexuality studies

For cell and colony morphology analysis, 2 to 3-day-old cultures grown on MYP agar (g L^{-1}: yeast extract 0.5, malt extract 7, soytone 2.5, and agar 20) were used. The size of 20 randomly selected cells was estimated using an Olympus BX40 microscope, and, for each strain, average measures as well as standard deviations were calculated. Conventional assimilation and fermentation tests were assayed as described by Yarrow [33]. Assimilation of aldaric acids and aromatic compounds was also studied as described by Fonseca [8] and Sampaio [21], respectively. The ability to form the sexual stage was assessed with previously described procedures [13]. After 1-week incubation at 17–20°C, *X. dendrorhous* sexual structures were observed and photographed using a Leica DMR microscope, and a Leica DFC320 camera, respectively.

Molecular characterization

The micro/minisatellite primed-PCR technique (MSP-PCR) was used for PCR fingerprinting studies. DNA extraction, PCR and electrophoresis conditions, and gel image analysis procedures were those reported by Libkind et al. [15]. The primers employed were M13 (5′-GAG GGT GGC GGT TCT-3′) for minisatellite PCR experiments, and synthetic oligonucleotides $(GTG)_5$ and $(GAC)_5$ for microsatellite PCR experiments.

Biochemical studies

Carotenoid pigments

Five milliliter of an aqueous suspension (A_{580} = 0.5) of 48 h old yeast culture grown on PDA slants were used to inoculate 500-ml flasks containing 100 ml of carotenoid production medium (CPM), as described by Buzzini and Martini [4]. These flasks were then incubated at 21°C in an oscillatory rotary shaker at 150 rpm. Batch fermentations were carried out for 5 days; pH was not adjusted during the process. Cell dry weight was determined at 105°C until constant weight.

Cells from each flask were harvested by centrifugation at 12,000 rcf, and washed twice with distilled water. The resulting pellet was suspended in 3 ml dimethyl sulfoxide (DMSO, Merck). After vortexing vigorously, the pellets were placed in a water bath at 50°C for 1 h, to increase extraction efficiency. The colored DMSO suspensions were centrifuged (12,000 rcf, 20 min, 4°C) and DMSO treatment was repeated several times. Acetone (3 ml) was then added to the pellets, the mixture was vortexed and centrifuged, and the procedure was repeated until the yeast pellets lost all pigmentation. The pigmented supernatants were then pooled, and diluted with 10–15 ml water. Carotenoids were successively extracted with 5 ml of light petroleum ether (boiling range 40–60°C) until disappearance of pigmentation from the lower phase. When necessary, a few ml of saturated NaCl solution were added to improve phase separation. The combined organic phases were washed several times with water, dried over Na_2SO_4, filtered and vacuum concentrated. The extraction process was carried out under subdued light in order to avoid pigment degradation. The crude extracts were kept in sealed vials, under nitrogen atmosphere, at −20°C for future spectrometric analysis.

For UV-visible spectra studies, carotenoid extracts were mixed with known volumes of methanol, and analyzed in a Hewlett Packard P 8453–E UV–Vis spectrophotometer using 1-cm quartz Suprasil cuvettes. Astaxanthin was measured at 485 nm and expressed as μg of total carotenoids per L of culture broth (μg L^{-1}) or per g of dry biomass (μg g^{-1}) using specific extinction coefficient ($E^{1\%}_{1\ cm}$=2,100 [7]) and equation described by Schiedt and Liaaen-Jensen [23].

Carotenoid fraction of the extracts was resolved by reverse phase HPLC (Akta Basis, Amersham) employing a Phenomenex Gemini C18 (5 μm) column (with pre-column) and a UV–Vis detector set at 420, 450 and 500 nm. The solvent system consisted of solvent A [methanol/water

solution of 0.001 M tetrabutyl ammonium sulphate and 0.001 M of propionic acid (80:20)] and B [acetone/methanol (60:40)]. The flow rate was 1 ml min^{-1}. Gradient program consisted of an initial step at 85% of solvent A, which reached 100% of solvent phase B after 30 min, where it was maintained for additional 20 min. A standard sample of astaxanthin (Roche) was used as reference. The carotenoid pigments extract of *P. rhodozyma* Type strain (CBS 5905^T) was used for comparison in HPLC and UV–visible spectra studies.

Fatty acid composition

Biomass for fatty acid analysis was obtained by inoculating 250-ml flasks containing 100 ml of MMS broth [g L^{-1}: glucose 10, $(NH_4)_2SO_4$ 2, $KH_2(PO_4)$ 2, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCl_2 \cdot 2H_2O$ 0.1, and yeast extract 1] with 48 h old cell culture suspensions (5% v/v) grown on the same medium. The flasks were incubated in a rotary incubator shaker (Innova 4000, New Brunswick) at 250 rpm, for 96 h at 21°C. Biomass was harvested by centrifugation, thoroughly washed with distilled water, lyophilized and kept at −20°C.

Fatty acid methyl esters (FAME) were obtained in a three-step process: extraction, derivatization, and quantification on a gas chromatograph (GC). The procedures used were those of Zellmer et al. [34], with the following modifications: helium was used as the carrier gas. Results are reported as μg FAME mg^{-1} of dry biomass (db).

Extracellular enzymes

Because composition of the culture medium used to obtain cell inoculums may affect the production of many extracellular enzymes (for example, a rich medium may repress some activities), three culture media were tested in this work. These were YM (g L^{-1}: yeast extract 3, malt extract 3, peptone 5, dextrose 10, and agar 20), MYP and PDA. Standardized suspensions (10^6 cell mL^{-1}) of 48 h old cultures of CRUB 1149 and CBS 5905^T grown at 18°C on each of the three media cultures were used to inoculate two sets of Petri dishes. The first set was incubated at 5°C for 21 days, while the second set was incubated at 20°C for 5 days. Amylase, protease, esterase, cellulose, pectinase and pectate-lyase activities were measured as described by Brizzio et al. [3] with modifications. Cellulose activity was tested on media composed of (g L^{-1}): carboxymethylcellulose 10, cellobiose 0.5, YNB 6.7, and agar 20. After incubation, plates were flooded with a 0.03% Congo red solution for 15 min, and washed with 1 M ClNa. Pectate lyase activity was tested using the same media as for pectinase, but at pH 5. The extracellular enzymatic production was estimated semi-quantitatively by the colony/halo diameter ratio. Because this ratio was homogeneous for positive enzyme activities in *X. dendrorhous* for the three tested media, average values and standard deviations were used. Enzymatic activity between both the strains was compared using student's *t* test.

Results and discussion

In this work, CRUB 853 and CRUB 1149 were selected as representative strains of the new population of *X. dendrorhous* recently discovered in Patagonia [16]. As previously stated, the first strain was isolated from the stromata of *Cyttaria hariotii*, while the second strain (CRUB 1149) was isolated from a mountain lake. It is believed that the stromata of *C. hariotii* is the original habitat of *X. dendrorhous* in Patagonia, while the presence of this yeast in the lake could be explained by the presence of *Cyttaria*-containing *Nothofagus* trees in the surrounding area, and that when mature, stromata fall into the water.

Phenotypic characterization and sexual studies

Morphological characterization of these strains and comparison with reference strains (Type strains of *Xanthophyllomyces dendrorhous* CBS 7918^T and of *Phaffia rhodozyma* CBS 5905^T) revealed almost no heterogeneity. Cells were in all cases spherical to ovoid. Patagonian yeast cells were slightly smaller (3.5–4.4 μm × 3.4–3.9 μm) than the Type strain cells (4.7–5.1 μm × 3.6–3.7 μm). However, these differences were not statistically significant. No differences were observed in colony morphology, except for the fact that orange pigmentation in Patagonian strains was less intense than Type strains. The results of physiological and biochemical tests showed that Patagonian strains had a similar assimilation profile to Type strains, differing only in the assimilation capacity of some carbon (inulin, ribitol and xylitol) and nitrogen (L-lysine and cadaverine) sources (Table 1), and in the ability to ferment raffinose. Sexuality studies in polyol-containing media revealed that Patagonian strains produced sexual structures after the conjugation of a mother cell and its bud (pedogamy). This conjugation type is typical of *X. dendrorhous* [10]. The result was the formation of slender holobasidia of 2–3.5 μm wide and 50–170 μm length (Fig. 1). Frequently, three to four thin-walled spores (oval or ellipsoidal) were terminally produced on the apex of the basidium (Fig. 1). The morphological characteristics of the sexual stage of the Patagonian strains were similar to those of the Type strain CBS 7918^T. As expected, strain CBS 5905^T did not produce sexual structures [6, 13].

Table 1 Physiological characterization of Patagonian vs. collection *X. dendrorhous* strains

Tests	*X. dendrorhous* strains		
Carbon sources	Coll[a]	CRUB 0853	CRUB 1149
D-Glucose	+	+	+
D-Galactose	–	–	–
L-Sorbose	–/+w	+w	–
D-Glucosamine	–	–	–
D-Ribose	+w/–	–	–
D-Xylose	+s	+s	+s
L-Arabinose	+	+	+
D-Arabinose	–	–	–
L-Rhamnose	–	–	–
Sucrose	+	+	+
Maltose	+	+	+
α,α-Trehalose	+	+	+
Methyl-α-D-glucoside	–	–	–
Cellobiose	+	+	+
Salicine	+s	+s	+sw
Melibiose	–	–	–
Lactose	–	–	–
Raffinose	+	+sw	+
Melezitose	+	+s	+s
Inulin	–	+sw	+s
Soluble starch	+	+	+
Glicerol	+s	+s	+sw
Erythritol	–	–	–
Ribitol	+w	–	–
Xilitol	+w	–	–
D-Glucitol	+w	+	+
D-Mannitol	+	+	+
Galactitol	–	–	–
Myo-inositol	–	–	–
Glucono-δ-lactone	+s	+s	+s
D-Gluconic acid	ND	+sw	+sw
D-Glucuronic acid	ND	–	+sw
D,L-Lactic acid	+s	+sw	+sw
Succinic acid	+s	+	+
Citric acid	+s	–	+sw
Methanol	–	–	–
Ethanol	+s	+s	+s
L-Malic acid	ND	+sw	+
L-Tartaric acid	ND	–	–
D-Tartaric acid	ND	–	–
m-Tartaric acid	ND	–	–
Saccharic acid	ND	–	–
Mucic acid	ND	–	–
Vanillic acid	ND	–	–
Veratric acid	ND	–	–
Ferulic acid	ND	–	–
p-Hydroxibenzoic acid	ND	–	–
m-Hydroxibenzoic acid	ND	–	–
Protocatechuic acid	ND	–	–
Catechol	ND	–	–
Gallic acid	ND	–	–
Salicylic acid	ND	–	–
Gentisic acid	ND	–	–
Phenol	ND	–	–
Nitrogen sources			
Potassium nitrate	–	–	–
Nitrite	–	–	–
Ethylamine	–	–	–
L-Lysine	+w	–	–
Cadaverine	+w	–	–
Creatine	–	–	–
Creatinine	–	–	–
D-Glucosamine	ND	–	–
Fermentation			
Glucose	+	+	+
Galactose	ND	–	–
Maltose	+w	+s	+s
Methyl-α-D-glucoside	ND	–	–
Sucrose	+w	+s	+
Trehalose	+w	+s	+s
Melibiose	ND	–	–
Lactose	ND	–	–
Cellobiose	+w	+s	–
Melezitose	+s	+s	+s
Raffinose	+w	–	–
Inulin	ND	–	–
Xylose	ND	–	–
Other tests			
Growth w/vitamins	–	–	–
0.01% cicloheximide	–	–	–
0.1% cicloheximide	–	–	–
Growth at 20°C	+	+	+
Growth at 25°C	+w	+w	+w
Growth at 30°C	–	–	–
Amilaceous compounds	+	+	+
Urea hydrolysis	+	+	+
DBB	+	+	+

+ Positive, − negative, *w* weak growth, *s* slow or delayed growth

[a] Collection strains, data obtained from Golubev [10]

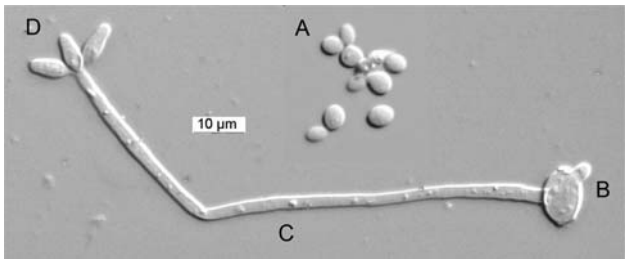

Fig. 1 Sexual structures of Patagonian *X. dendrorhous* strain CRUB 853. *A* vegetative cells, *B* daughter cell-mother cell conjugation (pedogamy), *C* holobasidium, *D* terminal basidiospores

Molecular characterization

The MSP-PCR fingerprinting using three different primers showed that Patagonian strains form a genetically homogeneous group (regardless of the substrate of origin) (Fig. 2). This group is genetically different from the Type strains. Libkind et al. [16] also found genetic differences in MSP-PCR fingerprints between *X. dendrorhous* Patagonian and Type strains but using only one of the three primers used in the present work. Moreover, such publication reported the absence of variability between the strains in the D1/D2 region of the 26S rDNA, whereas the ITS region did in fact separate Patagonian strains in a distinct cluster. The authors related the observed polymorphisms, for the species in general, to host specificity, i.e. different *X. dendrorhous* lineages apparently colonize different tree species or tree families. In the case of Patagonian strains, it is hypothesized that the association of *Xanthophyllomyces* with *Cyttaria* is derived from previous yeast, *Nothofagus* association. To test this hypothesis, an extended survey to determine the distribution of *X. dendrorhous* in different *Nothofagus* species, and in different locations in Patagonia, is currently under way by our laboratory.

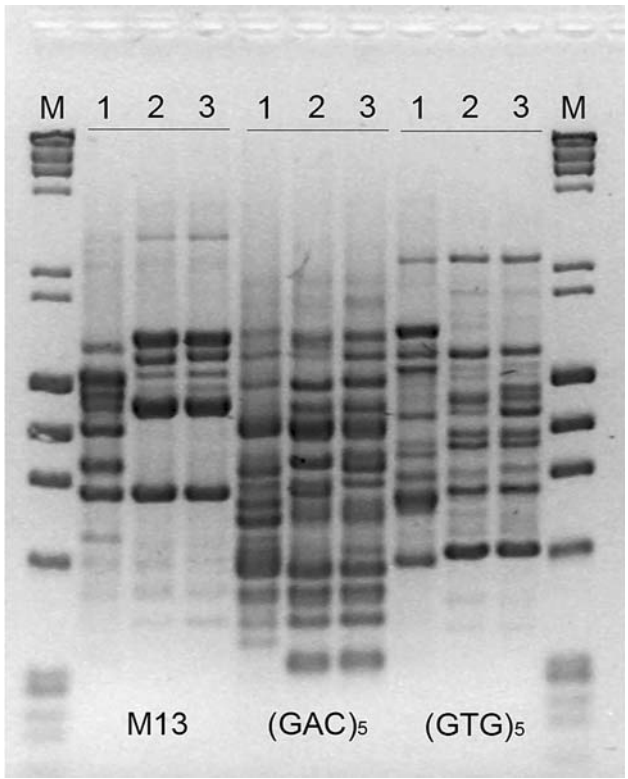

Fig. 2 MSP-PCR DNA profiles of *X. dendrorhous* Type strain CBS 7918^T (*1*), and Patagonian strains CRUB 853 (*2*) and CRUB 1149 (*3*). *M* molecular size marker (*λ* DNA cleaved with *Hin*dIII and ΦX174 DNA cleaved with *Hae*III)

Biochemical studies

The new population/phylotype of *X. dendrorhous* was studied to assess its biotechnological potential in terms of astaxanthin, fatty acids and extracelullar enzymes production.

Carotenoid composition

Patagonian CRUB 853 and CRUB 1149 strains produced 70.6 ± 2.1 and 145.5 ± 11.6 µg g^{-1} db of total carotenoids, respectively. *X. dendrorhous* wild strains produce low concentrations of carotenoid pigments (less than 300 µg g^{-1}) [1,7]; however, carotenoid content of Patagonian strains was particularly low. This could explain the weaker colony color compared to collection strains. Strain CRUB 1149 produced two times more carotenoids than CRUB 853. This may be related to the origin of the strains, as CRUB 853 was found in a high altitude lake exposed to higher UV radiation than the lowland forest from which CRUB 1149 was isolated. This hypothesis is consistent with the belief that carotenoids have a photoprotective role in yeasts [26] and is also in agreement with the antioxidant activity of astaxanthin [24,25].

Carotenoid extract UV–Vis spectra had a broad spectral band with no evident absorption peaks, and a maximum at 467 nm (Fig. 3a). HPLC studies revealed the presence of a major pigment (≈80% of total carotenoids) with a retention time of 20.76 min (Fig. 3b), very similar to that of the astaxanthin standard (21.24 min). Furthermore, HPLC analysis using astaxanthin containing *P. rhodozyma* CBS 5905^T showed retention time for this pigment identical to that of Patagonian *X. dendrorhous* strains.

According to the UV–Vis spectra, xanthophyll-like pigments predominated in the Patagonian strains [31], and were similar to those of the Type strain, indicating a similar carotenoid composition. The proportion of the major pigment detected by HPLC in the Patagonian strains coincides with the usual proportion of astaxanthin reported for *X. dendrorhous* [1, 2]. Confirmation of the presence of astaxanthin in Patagonian strains is obtained from the comparison with the Type strain HPLC chromatogram. According

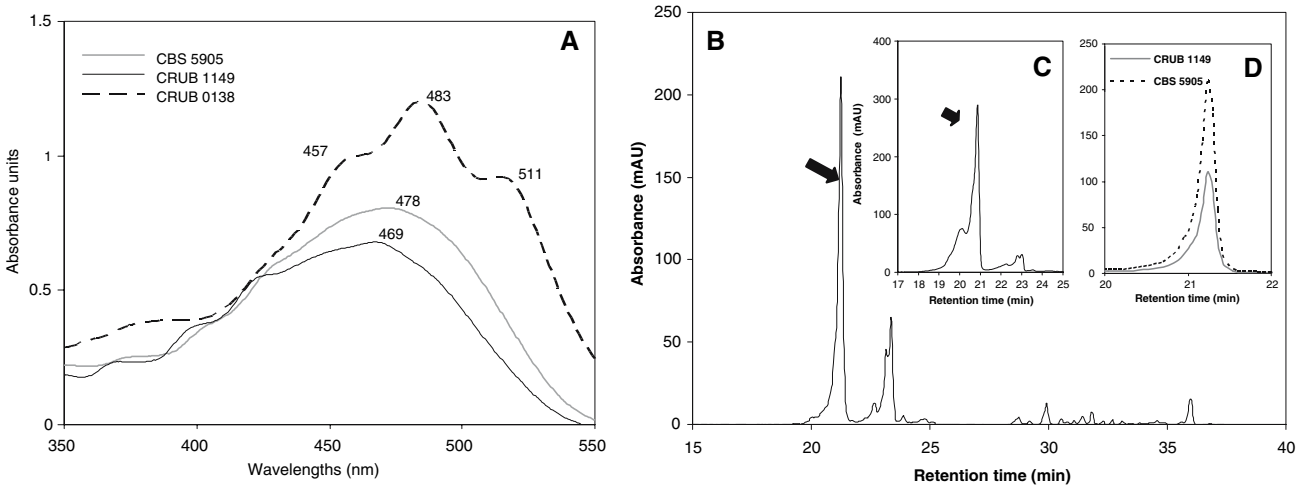

Fig. 3 UV-visible spectrophotometric and HPLC analyses of carotenoid pigments from *X. dendrorhous* CRUB 1149 and CBS 5905^T. **a** UV-visible spectra of both Patagonian and Type strains of *X. dendrorhous* and comparison with a nonastaxanthin producing yeast: *Rhodotorula mucilaginosa*. **b** HPLC chromatogram of CRUB 1149 extracts. *Arrow* indicates peak corresponding to astaxanthin. **c** HPLC chromatogram of CRUB 1149 extracts supplemented with astaxanthin standard. *Arrows* indicate peaks corresponding to astaxanthin standard. **d** Comparison of astaxanthin peak of CRUB 1149 and CBS 5905^T

to the data obtained, it was concluded that Patagonian isolates produce astaxanthin as the major carotenoid pigment, and they do not differ in this physiological trait from the rest of the *X. dendrorhous* strains.

Fatty acids composition

Fatty acids (FA) of strain CRUB 853 represented a small proportion of total dry biomass (3.64 ± 0.15%) compared to FA content of other pigmented species like *Rhodotorula* or *Sporobolomyces* (10–20%) [18]. The major FA produced by CRUB 853 was linoleic acid (C18:2n6c) an essential ω-6 type lipid (64.8%), followed by oleic acid (C18:1n9c) (15%), palmitic acid (C16:0) (11.3%) and α-linolenic acid (C18:3n3) (5.4%). These FA have been found in other yeast species [18, 27–29], and also have been detected in *X. dendrorhous* [22]. Other FA found in CRUB 853 in trace quantities (<2%) were stearic (C18:0), undecanoic (C11:0), pentadecanoic (C15:0), palmitoleic (C16:1n7), heptadecanoic (C17:0), γ-linolenic (18:3n6) and eicosatrienoic (C20:3n3) acids.

Despite the low amount of FA produced by CRUB 853, it is interesting to note the high proportion of polyunsaturated fatty acids (PUFAs, 70.5%) present in the Patagonian strain. This proportion is unusual in yeasts, as they normally contain 20–30% PUFAs [18, 22, 35], and is much higher than that reported for *X. dendrorhous* commercial strains [22]. Higher amounts of PUFAs have been found for Antarctic yeasts, though they rarely exceed 50% of total FA [28, 29]. FA composition in yeasts is largely affected by the C/N ratio of the culture medium. Thus, future studies should focus on the optimization of culture conditions for an enhanced lipid production in *X. dendrorhous* Patagonian strains, and on assessment of the potential utilization of these strains as a biotechnological source of FA, in particular PUFA.

Extracellular enzyme activity

Extracellular enzymes synthesis and effects of temperature on activity were similar for all the three culture media studied, and two strains tested. Of the six enzymes studied, only amylase activity was detected, both in Patagonian and Type strains. The halo/colony ratio showed that the Patagonian strain had significant (P<0.001) higher amylase activity (3.44 ± 0.21) than the Type strain (2.09 ± 0.24). Amylase activity has been previously reported for *X. dendrorhous* collection strains [5]. The authors characterized this enzyme as β-amylase, and highlighted its potential applications in the production of maltose syrup and, especially in the beer industry. In the present study, amylase activity was found only at 4°C, suggesting that the synthesis of such enzyme in *X. dendrorhous* is favored by low temperatures.

Conclusions

We characterized the strains of a unique Patagonian population of *X. dendrorhous* at a morphological, biochemical, physiological and genetic level. Patagonian strains did not differ significantly from Type strains in their phenotypic characteristics. Only a few minor physiological differences were detected. As shown by the MSP-PCR studies reported, most differences between the Patagonian and

collection strains are at the genetic level. These differences could be explained by the geographic isolation and the unique habitat of the Patagonian population.

Carotenoid pigment analysis showed that Patagonian isolates produce a xanthophyll-type pigment, most likely astaxanthin, just as their co-specific strains from the North hemisphere do. According to the biochemical studies, fatty acids, mainly of polyunsaturated nature were found in Patagonian *X. dendrorhous*. Amylase extracellular hydrolytic activity was detected in *X. dendrorhous*. This enzyme has been reported to have certain potential industrial applications. Thus, Patagonian *X. dendrorhous* strains proved to be a potential source of several biotechnological important compounds.

This report contributes to understand the biological and biochemical variation of *P. rhodozyma*/*X. dendrorhous*, which is relevant for biotechnological research as strains with interesting properties might be unveiled.

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