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Isolation of Carotenoid-producing Yeasts from an Alpine Glacier

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Cold-adapted yeasts are increasingly being isolated from glacial environments, including Arctic, Antarctic, and mountain glaciers. Psychrophilic yeast isolates mostly belong to *Basidiomycota* phylum, such as *Cryptococcus*, *Mrakia*, and *Rhodotorula*, and represent an understudied source of biodiversity for potential biotechnological applications. Since some basidiomycetous yeast genera (e.g. *Rhodotorula*, *Phaffia*, etc.) were demonstrated to produce commercially important carotenoids (e.g. β-carotene, torulene, torularhodin and astaxanthin), the present study aimed to obtain psychrophilic yeast isolates from the surface ice of an Italian glacier to identify new pigment-producers. 23 yeast isolates were obtained. Among them, three isolates giving pigmented colonies were subjected to ITS1/ITS2 sequencing and were attributed to the Basidiomycetous yeasts *Dioszegia* sp., *Rhodotorula mucilaginosa*, and *Rhodotorula laryngis*. The strains were cultured batch-wise in a carbon-rich medium at 15°C until the stationary phase was reached, then the pigments were extracted from freeze-dried biomass using a DMSO:acetone mixture. Visible absorption spectrum and HPLC-DAD analysis revealed the presence of carotenoid pigments. In batch cultures of *Dioszegia* sp., carotenoid production was growth-associated and yielded up to 3.4 mg/L of a molecule exhibiting an *m/z* ratio (568) consistent with the molecular weight of xanthophylls bearing 2 OH groups.

1. Introduction

Cold adapted yeasts, which are capable to survive and thrive at low and/or subzero temperatures, are continuously being isolated from permanently cold ecosystems, including polar and mountain glaciers (Butinar et al., 2007; Rossi et al., 2009; D'Elia et al., 2009; Margesin, 2009). The ecological role of yeasts in glacial environments remains to be deciphered, as to whether they exert an active role in bio-geochemical cycles or they are just preserved in metabolically inactive status. The biodiversity of these organisms is still understudied and underestimated as well, mostly due to the lack of studies using culture-independent approaches aiming to identify non-cultivable strains. Nowadays, most isolates belong to the fungal phylum of *Basidiomycota*, and particularly to the genera *Rhodotorula*, *Cryptococcus*, and *Mrakia*. These yeasts represent an invaluable source of biotechnological resources, in terms of cold active enzymes and unexplored metabolic potential. For example, it has been shown that oleaginous yeasts are very common among psychrophilic isolates and processes for lipid production with cold adapted species of *Rhodotorula* have been described (Amaretti et al., 2010).
A potential application of psychrophilic yeasts is the production of carotenoids. In effect, some basidiomycetous yeast genera (e.g. *Rhodotorula*, *Phaffia*, etc.) were demonstrated to produce commercially important carotenoids (e.g. β-carotene, torulene, torularhodin and astaxanthin) and are referred to as red yeasts (Frengova and Beshkova, 2009). Carotenoids are a family of tetraterpenoids bearing an extensive conjugated polyene system, responsible for absorption of blue light and for their yellow to red color (Fraser et al., 2004). They can be classified as carotens, which are hydrocarbons (such as lycopene, torulene, and α to ε-carotene), or xanthophylls, which bear hydroxyl, keto, and/or carboxyl groups (such as zeaxanthin, lutein, astaxanthin, and torularhodin) (Figure 1). Carotenoids are widespread in nature, being produced as photoprotectants by plants, some photosynthetic bacteria, and some fungi. They are of great interest for food, pharmaceutical, and cosmetic industries, where they find application as vitamin A precursors, pigments, antioxidants, and/or photoprotectants (Fraser and Bramley, 2004).

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Furthermore, they are attracting considerable attention for application in nutraceuticals, and cosmeceuticals, since they exert a number of beneficial effects on health, including maculoprotective, anti-oxidant, anti-inflammatory and anti-tumoral properties (Fraser and Bramley, 2004; Sommer et al., 2012; Rasmussen et al., 2013). In the last decades the increasing demand for natural carotenoids has focused attention on the development of biotechnological processes, alternative to chemical synthetic methods following extraction from plants. Therefore, fermentative processes exploiting the so-called red yeasts are increasingly being described (Nelis et al., 1991; Wang et al. 2007; Frengova and Beshkova, 2009; Moliné 2012).
The present study aimed to obtain new isolates of environmental red yeasts, in order to identify new potential sources for biotechnological production of carotenoids. In this perspective, the yeast biodiversity contained in a surface ice sample from an Alpine glacier was investigated for carotenoid production, because the phylum to which most red yeasts belong, *Basidiomycota*, is particularly frequent among psychrophilic fungi.

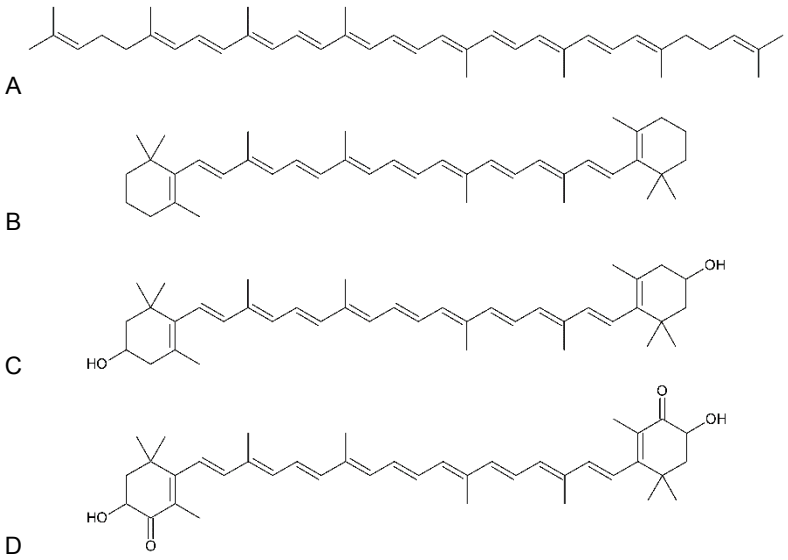

Figure 1: Structure of carotenes (A, B) and xanthophylls (C, D): lycopene (A), β-carotene (B), zeaxanthin (C), astaxanthin (D).

2. Materials and methods

2.1 Yeast isolation and cultivation

Surface ice samples were aseptically collected from an Italian alpine glacier (Tonale Pass, TN, Italy) into sterile containers. Ice samples were allowed to melt at 4°C. 50 mL of melt liquid were centrifuged (5000 rpm, 4°C) to concentrate solids to 5 mL. 0.2 mL of the suspension were plated onto GMY (40 g/L glucose, 3 g/L yeast extract, 8 g/L KH_2PO_4) agar plates supplemented with 50 mg/L chloramphenicol (CAF). Plates were incubated at 5°C until colonies were observed. Colonies with yeast morphology were seeded onto the surface of GMY agar slants and maintained at 4°C. Pigmented yeasts were cultured in shaken flasks of YPD (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract) and GMY broth at 5 or at 15°C.
Bioreactor batch cultures were performed in a laboratory-scale bioreactor (Labfors, Infors, Bottmingen, Switzerland) containing 2.5 L of GMY medium. The bioreactor was inoculated 10% v/v with a 48-h seed-culture grown in GMY. The culture was kept at 15°C, aerated with 1 v/v/min air, and stirred at 300 to 700 rpm to keep the DOT at 20%. Samples were collected periodically to monitor the growth and to analyze glucose and carotenoids. Biomass dry weight (DW) was determined gravimetrically; glucose was analyzed by HPLC-RID (Amaretti et al. 2010). Biomass/glucose yield coefficient ($Y_{X/S}$) was calculated as the ratio between biomass generated and glucose consumed. The specific growth rate (μ) was calculated by least square regression in the linear tract of the semi-logarithmic growth curve.
All chemicals were purchased from Sigma-Aldrich (Steinnheim, Germany), unless otherwise stated.

2.2 Taxonomic identification

Yeast biomass was collected from 1 mL of grown YPD cultures by centrifugation (10,000 rpm, 5 min, 4°C), was washed twice with water, and was subjected to cell lysis and extraction of genomic DNA using a commercial kit (Master Pure Yeast Dna Purification Kit, Epicentre, Madison, Wisconsin), according to the

manufacturer's protocol. 1 µL of genomic extract was utilized as template for PCR amplification of ITS1 and ITS2 regions within rDNA genes (White et al., 1990). Amplicons were sequenced (Eurofins MWG Operon, Ebersberg, Germany), then taxonomic identification was performed through a comparison with GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed 12.12.2013).

2.3 Pigment extraction and analysis

Biomass was collected from 40 mL samples of GMY cultures (centrifugation at 10,000 rpm, 5 min, 4°C), washed twice with water, and frozen at -80°C. Biomass was lyophilized and extracted under agitation for 16 h at r.t. with a mixture of DMSO and acetone (1:1, v/v). The visible spectrum of the extracts was acquired in the range between 350 and 700 nm. Extracts were analyzed with a HPLC apparatus (1200, Agilent Technology, Santa Clara, CA, USA) equipped with a C18 column and a diode array detector (DAD). Elution was performed at r.t. with 1 mL/min of an isocratic mixture of acetonitrile, THF and water (5:3:2, v/v/v) (Masino et al. 2008). Quantification was performed with HPLC-DAD (λ = 450 nm), using β-carotene as external standard. Information about the molecular weight of carotenoids was obtained with HPLC coupled to a mass spectrometer detector (ESI ion trap).

2.4 Statistical analysis

Experiments were carried out in triplicate. Means were compared with Student's t-test and were considered different for $P < 0.05$.

3. Results and Discussion

3.1 Isolation of psychrophilic pigmented yeasts and growth properties

A sample of surface ice from an Italian alpine glacier was melted and plated onto CAF-supplemented GMY plates. 23 morphologically different yeast colonies arose within 10 d of incubation at 5°C. Three of them, N12, N22, and N23, presented orange to salmon-colored pigmentation and were selected for investigation of carotenoid production. These strains were subjected to amplification and sequencing of ITS1 and ITS2, the hyper variable regions spacing 18S, 5.8S, and 28S rDNA genes. The ITS sequences of N12, N22, and N23 presented high similarity (> 96%) with database ITS sequences from strains of *Dioszegia* sp., *Rhodotorula mucilaginosa*, and *Rhodotorula laryngis*, respectively. Diverse species of *Dioszegia*, and both *R. mucilaginosa*, and *R. laryngis* were already found in polar or mountain glacier-associated habitats (Butinar et al., 2007; Rossi et al., 2009, Connell et al., 2010). *Dioszegia* sp. N12, *R. mucilaginosa* N22, and *R. laryngis* N23 were cultured in aerobic flasks of GMY medium, in order to determine their ability to grow at different temperatures. All the strains behaved as psychrophiles, since they grew abundantly at 5°C and were unable to grow at 30°C. Despite being psychrophiles, the yeasts may be utilized in a biotechnological process, since they were capable of growing at feasible temperatures for technological application (15 and 18°C). All the strains exhibited higher µ ($P < 0.05$) at 15°C compared with 5°C. In *R. mucilaginosa* N22 $Y_{X/S}$ was higher at 15°C than at 5°C ($P < 0.05$), whereas in *Dioszegia* sp. N12 and *R. laryngis* N23, it was similar ($P > 0.05$) at both the temperatures.

Table 1: Growth parameters of Dioszegia sp. N12, R. mucilaginosa N22, and R. laryngis N23 at 5 and 15°C. Values are means (n = 3, SD always < 10%); stars indicate statistically significant difference (P < 0.05).

	$Y_{X/S}$		µ, h^{-1}	
Strain	5°C	15°C	5°C	15°C
Dioszegia sp. N12	0.37	0.37	0.037	0.052*
R. mucilaginosa N22	0.24	0.58*	0.041	0.096*
R. laryngis N23	0.47	0.44	0.024	0.037*

3.2 Pigment extraction and analysis

The pigments of *Dioszegia* sp. N12, *R. mucilaginosa* N22, and *R. laryngis* N23 were successfully extracted with DMSO:acetone from the lyophilized biomass of stationary phase cultures and were subjected to visible spectroscopy in order to obtain preliminary information about the presence of carotenoids. In fact, carotenoids present characteristic visible absorption spectra, with a typical three-peak profile (or two peaks and a shoulder) between 400 and 550 nm, due to their highly conjugated double-bond systems (Young and Hamilton, 1999). The DMSO:acetone extracts obtained from the yeasts yielded visible absorption spectra comparable with that of β-carotene dissolved in the same solvents, but the three-peak pattern was

shifted to higher wavelengths (Figure 2). Thus, it was conceivable that the pigmentation of *Dioszegia* sp. N12, *R. mucilaginosa* N22, and *R. laryngis* N23 was due to the presence of carotenoids.

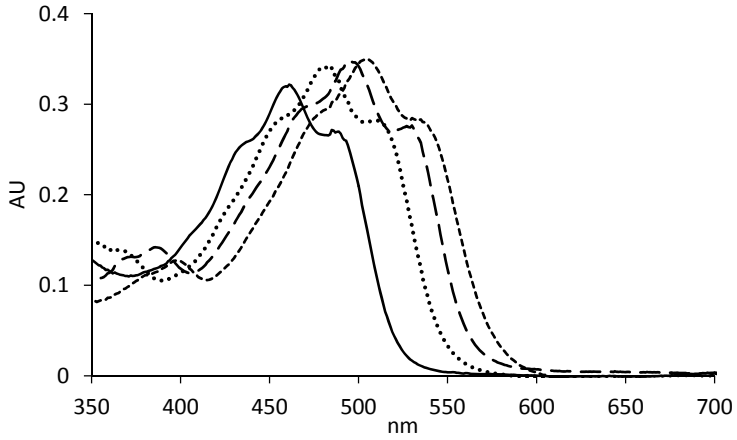

Figure 2: Visible spectrum of β-carotene (solid line) and of the organic extract of Dioszegia sp. N12 (dots), R. mucilaginosa N22 (short dashes), and R. laryngis N23 (long dashes).

The organic extracts of the yeasts were analyzed with reverse-phase HPLC-DAD (Figure 3). *Dioszegia* sp. N12 and *R. mucilaginosa* N22 yielded one major peak each, at 6.4 and 7.6 min respectively, which exhibited a spectrum similar in shape to a carotenoid (λ_{max} = 480 and 500 nm, respectively). *R. laryngis* N23 yielded 3 peaks, at 3.3, 3.6, and 3.7 min, each presenting a spectrum consistent with that of carotenoids (λ_{max} = 490, 480, and 485 nm, respectively). All these peaks were eluted before β-carotene (t_R = 20 min) and were presumably more polar.

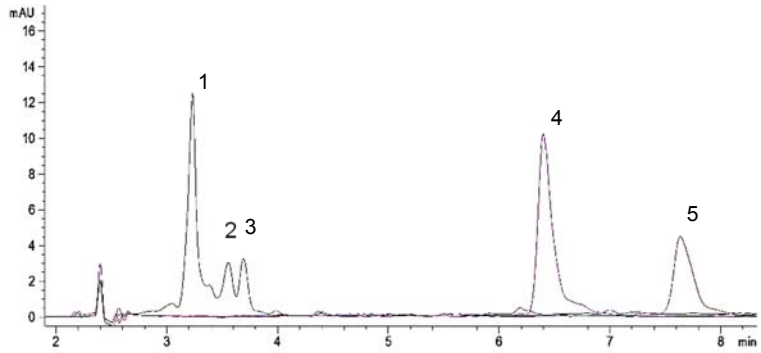

Figure 3: Overlaid HPLC-DAD chromatograms of the extracts from Dioszegia sp. N12 (peak 4), R. mucilaginosa N22 (peak 5), and R. laryngis N23 (peaks 1, 2, and 3). Chromatograms were registered at 450 nm.

3.3 Carotenoid production by *Dioszegia* sp. N12

Dioszegia sp. N12 was selected for deeper investigation of carotenoids production. HPLC-MS analysis revealed that this compound yielded a parent ion with *m/z* = 568, which is consistent with the molecular weight of xanthophylls bearing 2 OH groups (such as lutein and zeaxanthin). Nonetheless, it was excluded that the carotenoid produced by *Dioszegia* sp. N12 was either lutein or zeaxanthyn, since they were eluted with lower retention times. It is remarkable to note that production of xanthophylls other than astaxanthin is not common in red yeasts and has been described only in some species belonging to *Dioszegia* (Madhour et al. 2005). Therefore, the structure of the pigment yielded by *Dioszegia* sp. N12 deserves deeper structural investigation.

Bioreactor batch cultures were carried out at 15°C in order to determine growth and production kinetics (Figure 4). Compared with flask cultures, the strain grew with higher specific growth rate (μ = 0.076 h^{-1}, $P < 0.05$) likely due to improved oxygen transfer. Growth ceased being exponential after 48 h, then continued at lower rate until glucose got exhausted after 160 h. At the end of the fermentation, 17 g/L biomass were

produced, with an $Y_{X/S}$ of 0.43. Carotenoid production was growth associated and occurred in parallel with biomass generation. 3.4 mg/L carotenoid were produced at the end of the fermentation, which corresponded to a content of 0.2 mg carotenoid per g of biomass DW. The amount of carotenoid produced by *Dioszegia* sp. N12 is similar to that obtained in batch cultures with other wild type red fungi (Wang et al., 2007; Madhour et al., 2007; Frengova and Beshkova, 2009). These results make *Dioszegia* sp. N12 a potential candidate for carotenoid production. Thus, possible strain and process improvement deserve further investigating.

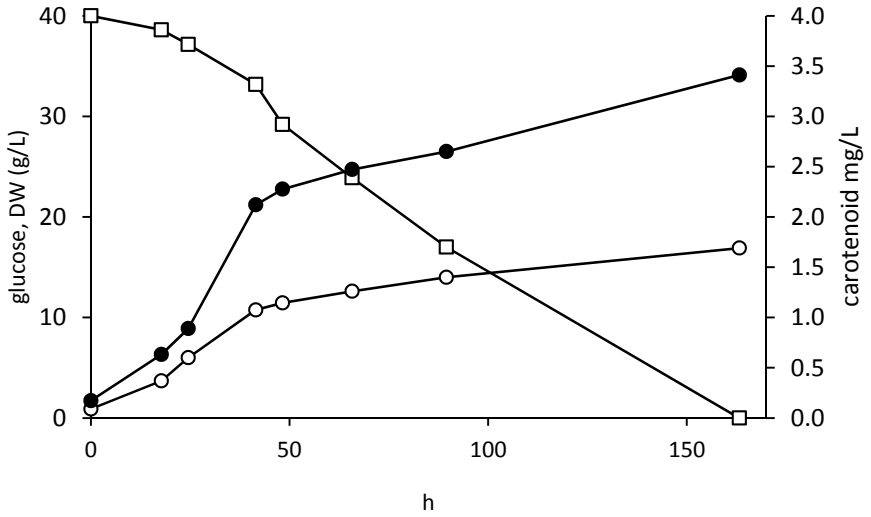

Figure 4: Time-course of glucose (square), biomass (empty circle), and carotenoid (filled circle) during a batch fermentation of Dioszegia sp. N12 in GMY medium. Triplicate experiments were carried out. A representative fermentation is reported.

4. Conclusions

This study confirmed that glacial environment are an underexploited source of biodiversity which deserves being investigated for ecological and biotechnological purposes. One of the new isolates produces a xanthophyll likely bearing two hydroxyl groups, which is quite uncommon among red yeasts. Therefore, this yeast is intriguing for deeper characterization of the carotenoid and for potential strains and/or process improvement.

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