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Genetic variability of astaxanthin-producing yeasts: random amplified polymorphic DNA (RAPD) analysis of Phaffia rhodozyma and Xanthopyllomyces dendrorhous

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ABSTRACT Astaxanthin (3,3ʹ-dihydroxy-β,β-carotene-4,4ʹ-dione)-accumulating yeasts are of great biotechnological interest. Random amplified polymorphic DNA (RAPD) analysis involving 5 primers and 13 astaxanthin-producing yeast strains was performed. Cluster analysis based on RAPD markers differentiated isolates of Xanthopyllomyces dendrorhous (self-sporulating) and Phaffia rhodozyma (asexual) at an intraspecific level. Strains considered to be derived from the same isolate, but which had had different strain histories, revealed significant differences in their RAPD patterns. The applicability of RAPD analysis for the species-level differentiation of these yeasts is discussed.

Phaffia rhodozyma was described by Miller et al. (1976). The accumulation of astaxanthin (3,3ʹ-dihydroxy-β,β-carotene-4,4ʹ-dione) as primary pigment in this red yeast created considerable biotechnological interest (Johnson and An 1991; Johnson and Schroeder 1996). Although astaxanthin is found in nature in several organisms (e.g. certain marine fish, crustaceans and birds), the number of natural sources of practical value is very limited. At the same time, both the aquaculture industry (as a feed supplement for salmon and trout) and various other applications connected with the excellent antioxidant properties of astaxanthin (Schroeder and Johnson 1996) have proved to be a rapid and very sensitive molecular method when genetic polymorphism is to be detected (Williams et al. 1990). This assay has been employed for the characterization of many fungi, e.g. Rhizomucor (Vastag et al. 2000), Gilbertella (Papp et al. 2003) and Rhizopus (Vágvölgyi et al. 2004).

The aim of the present study was to investigate the utility of DNA polymorphisms detected by RAPD analysis for astaxanthin-producing yeasts. Five random primers were tested for their ability to detect variability among 13 yeast strains, and cluster analysis was performed on the basis of these data.

Materials and Methods

Microorganisms, media and culture conditions

The names and origins of the 13 yeast strains examined are listed in Table 1. On the basis of their collection codes, some of the strains (1, 7, 8 and 10; 2 and 4) were regarded as originating from the same isolate, but they were obtained independently from different culture collections. Three strains of Cryptococcus sp. (designated Cr1, Cr69 and Cr76) were used as outgroups for numerical analysis. The strains were maintained on yeast-malt extract agar (YM: 0.5% malt extract, 0.25% yeast extract, 1% glucose, 0.25% peptone and 1.5% agar) slants at 4°C. For nucleic acid extractions, the strains were cultivated in liquid YM medium at 18°C with continuous shaking (200 rpm).

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Total DNA was isolated by a modification of the method of Leach et al. (1986). Yeast DNA sequences were amplified by the 10-mer primers OPC-02 (5ʹ-GTGAGGCGTC-3ʹ), OPC-04 (5ʹ-CCGCATCTAC-3ʹ), OPC-05 (5ʹ-GATGACCGCC-3ʹ), OPC-07 (5ʹ-GTCCCGACGA-3ʹ) and OPC-08 (5ʹ-TGGACCGTG-3ʹ) from Operon Kit C (QIAGEN Operon, Alameda, CA, USA).

Amplifications were performed as described earlier (Vastag et al. 2000), with slight modifications. The reaction mixtures (25 μl) contained 1 x PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂ and 0.1% Triton X-100), 200 μM each of the dNTPs (Pharmacia, Peapack, NJ, USA), 2.5 mM MgCl₂, 0.2 μM primer, 0.5 U of Taq DNA polymerase (Zenon, Oakville, ON, Canada) and 125 ng of genomic DNA. Samples were overlayered with 40 μl of sterile mineral oil (Sigma, Budapest, Hungary). Control reactions, without genomic DNA extract, were also run.

PCR was carried out with a PTC-100-60 DNA programmable thermal controller (MJ Research, Waltham, MA, USA) set for a denaturation step at 93°C for 1 min, followed by 45 cycles at 92°C for 1 min, at 37°C for 1 min and at 72°C for 1 min. An extension step at 72°C for 6 min was applied after the final amplification cycle.

The amplification products (10 μl of each reaction) were analysed by electrophoresis on 0.9% agarose gels in TAE.

Figure 1. Representative amplification patterns of Phaffia, Xanthophyllomyces and Cryptococcus strains obtained with OPC-02 used as primer. Lane 6, pUC Mix Marker DNA as size standard (Fermentas). Lane 13, HindIII-digested λ DNA as size standard (Fermentas). Lanes 1-5, strains 1, 2, 3, 4 and 5, respectively. Lanes 7-12, strains 6, 8, 9, 10, 11 and 12, respectively. Lane 14, strain 13. Lanes 15-17, Cryptococcus strains Cr1, Cr69 and Cr76, respectively.

Table 1. List of the astaxanthin-producing yeast strains investigated.

<table>
<thead>
<tr>
<th>Original code</th>
<th>Other code</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>CBS 5905</td>
<td>ATCC24202</td>
<td>ex Fagus crenata, Japan</td>
</tr>
<tr>
<td>CBS 5908</td>
<td>ATCC24203</td>
<td>ex Alnus japonica, Kiso, Japan</td>
</tr>
<tr>
<td>CBS 6938</td>
<td>CBC22365</td>
<td>ex sap on stump of Betula sp., Finland</td>
</tr>
<tr>
<td>ATCC 24203</td>
<td>CBS 5908</td>
<td>ex Alnus japonica, Kiso, Japan</td>
</tr>
<tr>
<td>ATCC 24229</td>
<td>-</td>
<td>ex Cornus brachypoda, Hiroshima, Japan</td>
</tr>
<tr>
<td>ATCC 24261</td>
<td>-</td>
<td>ex Betula maximowiciziana, Yamagata, Japan</td>
</tr>
<tr>
<td>CBS 5905</td>
<td>ATCC24202</td>
<td>ex Fagus crenata, Japan</td>
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<td>ex Fagus crenata, Japan</td>
</tr>
<tr>
<td>ATCC 24230</td>
<td>-</td>
<td>ex Betula papyrifera, Rainbow Lake, Alaska</td>
</tr>
<tr>
<td>CBS 5905</td>
<td>ATCC24202</td>
<td>ex Fagus crenata, Japan</td>
</tr>
<tr>
<td>ATCC 24230</td>
<td>-</td>
<td>ex Betula tauschii, Kiso, Japan</td>
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<tr>
<td>ATCC 24230</td>
<td>UC67-385</td>
<td>u.s., Norway</td>
</tr>
<tr>
<td>WIMP-UB</td>
<td>-</td>
<td>u.s., Norway</td>
</tr>
<tr>
<td>ZIMP-UB</td>
<td>-</td>
<td>-</td>
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</table>

*The code which is used throughout this paper for clarity.


Strains regarded as the same, but obtained independently from different sources: 1, 7, 8 and 10; 2 and 4.

RAPD analysis

Total DNA was isolated by a modification of the method of Leach et al. (1986). Yeast DNA sequences were amplified by the 10-mer primers OPC-02 (5ʹ-GTGAGGCGTC-3ʹ), OPC-04 (5ʹ-CCGCATCTAC-3ʹ), OPC-05 (5ʹ-GATGACCGCC-3ʹ), OPC-07 (5ʹ-GTCCCGACGA-3ʹ) and OPC-08 (5ʹ-TGGACCGTG-3ʹ) from Operon Kit C (QIAGEN Operon, Alameda, CA, USA).

The code which is used throughout this paper for clarity.

Other code

Source

1 CBS 5905 ATCC24202 ex Fagus crenata, Japan
2 CBS 5908 ATCC24203 ex Alnus japonica, Kiso, Japan
3 CBS 6938 CBC22365 ex sap on stump of Betula sp., Finland
4 ATCC 24203 CBS 5908 ex Alnus japonica, Kiso, Japan
5 ATCC 24229 - ex Cornus brachypoda, Hiroshima, Japan
6 ATCC 24261 - ex Betula maximowiciziana, Yamagata, Japan
7 CBS 5905 ATCC24202 ex Fagus crenata, Japan
8 CBS 5905 ATCC24202 ex Fagus crenata, Japan
9 ATCC 24228 - ex Betula papyrifera, Rainbow Lake, Alaska
10 CCY 77-1-1 CBS 5905 ex Fagus crenata, Japan
11 ATCC 24230 UC67-385 ex Betula tauschii, Kiso, Japan
12 WIMP-UB - u.s., Norway
13 ZIMP-UB - u.s., Norway
14 - - -
15 - - -
16 - - -
17 - - -

Figure 2. Dendrogram obtained by UPGMA linkage with clustering of Jaccard coefficients calculated from RAPD data. The scale represents dissimilarity (squared distance). The strain numbers on the left are those listed in Table 1. The cophenetic correlation coefficient of the similarity matrix and the resulting dendogram was 0.9825.
buffer (40 mM Tris-acetic acid pH 7.6, plus 1 mM Na₂EDTA) containing 0.5 μg/ml ethidium bromide. Banding patterns were visualized by UV fluorescence. HindIII-digested λ DNA (Fermentas, Vilnius, Lithuania) and pUC Mix Marker (Fermentas) were used as size standards. Each isolate was analysed by RAPD-PCR at least 3 times.

**Numerical analysis of the RAPD patterns**

The RAPD banding patterns were analysed in order to determine the genetic relatedness of the isolates. A matrix based on the presence or absence of amplicons observed after electrophoretic separation was created. From these data, similarity matrix of Jaccard coefficients were calculated and used with the UPGMA (unweighted pair-group method using arithmetic averages) linkage (Sneath and Sokal 1973) to produce a dendogram. Numerical analysis was performed with the SYNTAX 5.0 software package (Podani 1993).

**Results and Discussion**

All 5 primers used efficiently amplified various regions of the investigated yeast genomes. They revealed different levels of variability, but in general a substantial level of polymorphism was detected among the 13 strains. As an example, results of the RAPD experiment with the OPC-02 primer are shown in Figure 1.

The data derived from the RAPD experiments were used for cluster analysis. A dendrogram was generated by using unweighted pair group average linkage clustering of the Jaccard coefficients. The dendrogram obtained is shown in Figure 2. Three Cryptococcus strains were used as outgroups during this analysis. The dendrogram revealed 3 clusters (A, B and C) and 4 unclustered strains. Among these, we found the 3 Cryptococcus strains used as outgroups. They differed at a high level both from all Phaffia and Xanthophyllomyces strains and from each other. Cluster A contains 5 P. rhodozyma strains (strain 1 is the type strain of P. rhodozyma). Among them, 4 strains (1, 7, 10 and 8) are known to be the same origin, but had undergone prolonged maintenance in different culture collections. Suprisingly, their RAPD patterns displayed characteristic differences. Members of this cluster are not able to sporulate, but are able to produce a respiratory-deficient petite mutant (Kucsera et al. 1998) and have a genome size higher than 20 Mbp (Nagy et al. 1994). In contrast, cluster B involves Xanthophyllomyces strains with sporulation capability, a petite-negative character and a genome size around 16 Mbp (Nagy et al. 1994). Cluster C contains 2 Xanthophyllomyces strains with a common origin (ATCC and CBS maintenance), but with several different RAPD markers. Strain 13 was situated as an unclustered strain in the dendrogram.

These results prove the applicability of the RAPD method for investigations of intraspecific variability and for determinations of strain-specific markers (Phaffia and Xanthophyllomyces strains separate well when RAPD markers are evaluated), but they also demonstrate some limits of this approach. Particularly as concerns culture collection strains which have undergone prolonged maintenance, special care is required in assessments of natural genetic variability. Interestingly, some of the Xanthophyllomyces strains (strains 2, 4 and 13) also revealed very high, nearly species-level differences from the other Xanthophyllomyces strains in this RAPD analysis.

This rather high intraspecific genetic variability might originate from the aneuploid or polyploid state of these yeasts. An earlier study relating to the electrophoretic karyotyping of some wild-type and mutagenized strains of Xanthophyllomyces raised questions concerning their haploid character (Nagy et al. 1997). A recent investigation (Hermosilla et al. 2003) concluded that X. dendrorhous is diploid. A similar result was obtained when the ploidy of the P. rhodozyma type strain (ATCC 24202) was evaluated via flow cytometric analyses of propidium iodide-stained cells and mutagenic inactivation kinetics (Medwid 1998): the findings suggested that P. rhodozyma is polyploid.

The results of the present study provide further information concerning the genetic make-up of the two astaxanthin-producing yeasts P. rhodozyma and X. dendrorhous, and help to identify genetic markers for the species delimitation which is not allowed by simple morphological traits.

**Acknowledgments**

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**References**


