Research Paper

Isolation, characterization and long term preservation of mutant strains of *Xanthophyllomyces dendrorhous*

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The yeast *Xanthophyllomyces dendrorhous* is biotechnologically important due to its ability to produce the pigment astaxanthin, but is poorly understood at the genetic level. This is mainly because its preservation is difficult and many of the mutants obtained are unstable. The objectives of the present work were (i) the mutagenesis *X. dendrorhous* and, (ii) isolation of mutants with auxotrophic markers suitable for genetic studies of the carotenogenesis pathway and sexual cycle. Additionally, two kinds of preservation methods at the laboratory level were tested for the storage of strains. A collection of *X. dendrorhous* mutants affected in the production of carotenoid pigments or development of sexual structures and auxotrophic requirements were isolated by treatment with N-methyl-N′-nitro-N-nitrosoguanidine and the antibiotic nystatin. From a detailed analysis about the requirements of auxotrophic mutants the *ARG7*, *ARG3* and *PRO3* loci can be defined in this yeast. Among the methods assayed for the long-term preservation of *X. dendrorhous* strains, the dehydrated gelatin drop method showed the highest recovery of viable yeast after storage for 65 months. No changes in auxotrophic properties and in macro or micro morphology were observed after applying the latter method.

Keywords: Long-term storage / *X. dendrorhous* / Yeast preservation

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Introduction

Xanthophyllomyces dendrorhous (formerly *Phaffia rhodozyma*) is a fermentative basidiomycetous yeast able to grow in a variety of carbon sources, and is among the most promising biological sources for the commercial production of the pigment astaxanthin [1–7]. Astaxanthin has a high market price and a growing demand because it is used as additive for salmonid and poultry feeds to improve the coloration of flesh or egg yolks [8–11] and it may have a beneficial health effect on mammals through its powerful antioxidant properties [12, 13]. In spite of its biotechnological importance, little is known about the genetics of *X. dendrorhous,* and many efforts have been made to obtain mutants useful in classical genetic analysis of its carotenoid biosynthesis pathway and sexual cycle [1, 2, 14–18]. However the realization of these studies is a hard task, mainly because many of the mutants obtained are unstable and in general strains of *X. dendrorhous* are difficult to preserve for long periods of time. Currently, several methods to preserve microorganisms are available [19–21], where freeze drying is the most successful, yielding long viability, easy transport of strains, and protection against contamination [22, 23]. However, not all strains survive the process of freezing and for those that survive the viability may be very low [24, 25]. Furthermore the complexity, laboriousness and equipment required make them unsuitable for routine laboratory work.

The objectives of this work were the isolation of mutants of *X. dendrorhous* affected in the carotenogenic pathway, development of sexual structures with additional auxotrophic requirements useful as genetic markers. On the other hand, we tested two kinds of preservation methods applicable at the laboratory level

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for the long term storage of wild type and mutants strains of *X. dendrorhous*. The parameters evaluated were the recovery of cell viability, and conservation of morphological and auxotrophic characteristics of the strains.

Materials and methods

Yeast strains and culture conditions

X. dendrorhous strains ATCC 24230 (UCD 67-385) and CBS 7918 (VKM Y2786) and *P. rhodozyma* strain ATCC 24202 (UCD 67-210) were used as wild type. The strain S3-88 corresponds to a holobasidia-overproducing mutant derived from strain CBS 7918. Mutants affected in production of carotenoid pigments, development of sexual structures and additional auxotrophic requirements are listed in Table 1. The yeast strains were routinely grown at 22 °C in YM medium (1% glucose, 0.3% yeast extract, 0.3% malt extract, and 0.5% peptone). In the auxotrophic assays, minimal media (MMv) [18, 26] supplemented with the adequate requirements was used.

NTG mutagenesis and nystatin enrichment

Cells of *X. dendrorhous* were treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) [1] at a final concentration of 100 μg/ml for 30 min at 22 °C [17]. Cells were washed five times with 100 mM potassium phosphate, pH 7.0 and resuspended in YM medium. After incubation at 22 °C for 12 h, the cells were washed twice with MMv minimal media, resuspended in MMv and incubated for 5 h at 22 °C. Nystatin was added at a final concentration of 10 μg/ml and cells were incubated for 1 h at 22 °C with shaking at 40 rpm [17, 27, 28]. Finally, the cells were washed twice with YM medium, plated onto YM agar and incubated at 22 °C for 5–7 d. The screening for auxotrophic mutants was made by replica plate onto YM and MMv agar and those unable to grow on MMv were selected.

Cryopreservation methods

Sterile glycerol was added to 500 μl aliquots of late-exponential phase yeast cultures (4–5 d) to achieve 20% final concentration. These mixtures were treated by three different methods before storage at –70 °C: CRYI, freezing in liquid nitrogen for 20 min; CRYII, incubation at 4 °C for 2 h; and CRYIII, incubation at –20 °C for 24 h.

Preservation in dehydrated gelatin drops (DGD)

The method corresponds to that described by Marangon *et al*. [29] with few modifications. Colonies of strains were transferred to 500 μl YM broth to obtain a high density cellular suspension, and 500 μl of sterile 20% w/v GE0020 Gelatine powder (Scharlau Chemie) was added and mixed. Drops of 50 μl of this mixture were deposited onto the lid of a Petri dish covered with paraffin. When the gelatin drops hardened, the lid containing the drops was placed onto a base of a Petri dish filled with dehydrated silica gel and sealed. The plates were incubated at 22 °C until the gelatin drops were completely dry (48–72 h). The dehydrated drops were transferred into sterile 14 ml polystyrene Round-Bottom Tubes (Falcon) containing 10 ml of dehydrated silica gel under a cotton plug. The tubes were sealed with parafilm and maintained at 4 °C.

Quantification of cell viability

DGD method: one gelatin drop was dissolved in 1 ml of YM broth and serial dilutions were prepared. Aliquots of each dilution were plated onto YM agar plates and incubated at 22 °C until the development of visible colonies. The CFU/ml was determined and the percentage of survival was calculated in relation to the total cell number determined in a Neubauer chamber.

Cryogenic methods: tubes were chilled on ice and serial dilutions were prepared in YM medium. The cell number and percentage of survival were determined as described for the DGD method.

Morphological and auxotrophic analysis

Cells were streaked onto YM plates and incubated at 22 °C until the development of colonies (3 to 7 d). The morphological characteristics such as colony color and size, development of sexual structures, etc. were analyzed at the macro and microscopic level. To check the auxotrophic requirements, 200 μl of cellular suspension (10^7 cells/ml) of each strain were seeded onto an MMv agar plate. Once absorbed, sterile disks (5 mm diameter) were put onto this plate and 20 μl of sterile amino acid solution was deposited onto each filter. The plates were incubated at 22 °C for 5 to 7 d and the growth around each disk was recorded.

Sporulation assays

Cells were seeded onto MMv plates supplemented with 110 mM glucose and 28 mM ammonium nitrate, incubated at 22 °C for 2 d and then at 9 °C for 22 d, conditions that were previously reported as optimal for the induction of sporulation of *X. dendrorhous* [18]. The formation of sexual structures in each colony was inspected by optical microscopy (Zeiss model Axioskop2 microscope).

Results

Mutagenesis of *X. dendrorhous* and selection of mutants

Cells of strain CBS 7918 of *X. dendrorhous* were mutagenized using NTG and treatment with nystatin. Clones unable to grow on minimal medium (auxotroph) were selected and characterized in relation to specific auxotrophic requirements, color phenotype and development of sexual structures (Table 1). The same treatment was applied onto the prototrophic holobasidia-overproducing mutant S3-88, derived from *X. dendrorhous* CBS 7918, to obtain mutants with additional auxotrophic requirements. One mutant, A4-69, was unable to produce colored pigment (albino phenotype) and additionally showed arginine and methionine auxotrophy. The auxotrophic mutant A3-2 showed an interesting phenotype, because it is able to develop holobasidia, but unable to develop terminal basidiospores. This observation suggests that in the *X. dendrorhous* sexual cycle, the formation of holobasidia and basidiospores are independent events. In addition, the frequency of auxotrophic mutants obtained after nystatin enrichment from ATCC 24230 and CBS 7918 strains, calculated as the number of mutants obtained versus the total number of isolates analyzed, was 2.4×10^{-4} and 2×10^{-2} respectively (data not shown).

Preliminary definition of gene locus affected in auxotrophic mutants

To define the specific locus affected in *X. dendrorhous* auxotrophic mutants, these were analyzed for their ability to grow with intermediate products of the corresponding amino acid biosynthesis pathway. Five arg^- mutants derived from wild-type CBS 7918 require ornithine, citrulline or arginine to grow, suggesting that

Table 1. Strains of *X. dendrorhous* used in this work. Results obtained for morphological and auxotrophic characterization after preservation by the dehydrated gelatin drop method are shown.

Strains analyzed	Auxotrophic requirement	Phenotype	Source
Preserved for 27 months			
A1-55, A13-10, A21-100, A6-64, A3-71	orn or cyt or arg	arg^-	This work
A2-92, A10-68, A18-89	cyt or arg	arg^-	This work
A4-17, A7-35, A7-55, A12-85, A6-4	arg	arg^-	This work
A4-71, A12-20, A6-15	leu	leu^-	This work
A7-22, A12-32, A13-84	lys	lys^-	This work
A8-9, A12-74	ade	ade^-	This work
A8-47, A17-57, A18-90	met	met^-	This work
A12-82	asp	asp^-	This work
A3-2	leu	leu^-, bas^-	This work
A4-69	arg	arg^-, met^-, al	This work
Preserved for 38 months			
S3-88A1-24, S3-88A11-72, S3-88A15-2, S3-88A15-70, S3-88A2-86	lys	lys^-, hol^{++}	This work
S3-88A4-79, S3-88A7-2, S3-88A11-86	met	met^-, hol^{++}	This work
S3-88A5-64, S3-88A4-28, S3-88A6-13	ade	ade^-, hol^{++}	This work
S3-88A5-31, S3-88A10-23	arg	arg^-, hol^{++}	This work
S3-88A6-78, S3-88A11-87	leu	leu^-, hol^{++}	This work
S3-88A8-11	trp	trp^-, hol^{++}	This work
S3-88A9-32	asp	asp^-, hol^{++}	This work
S3-88A15-40	pro	pro^-, hol^{++}	This work
S3-88A8-74	ade, met	ade^-, hol^{++}	This work
Preserved for 65 months			
CBS 7919	nr	wt	
ATCC 24230	nr	wt	
NN385A13-21	ade	ade^-, red	[17]
atx5	nr	deep ylo	[14]
NNatx5A17-11	ade	ade^-, deep ylo	[17]
atx6	nr	ylo	[14]
NNatx6A10-6	cyt or arg	arg^-, ylo	[17]
atxS1	nr	red	[14]

Phenotypes: ylo, yellow; al, albino; bas^-, no production of basidiospores; hol^{++}, overproduction of holobasidia. ade, adenine; arg, arginine; cit, citrulline; met, methionine; orn, ornithine; leu, leucine; lys, lysine; asp, aspartic acid; trp, tryptophan; pro, proline; nr, no requirement. wt, wild type. The strain named A# and S3-88A#, correspond to mutants generated from CBS 7919 and S3-88 strains, respectively.

they are blocked in the conversion of N-acetyl-ornithine to ornithine. In *S. cerevisiae* this step is catalyzed by acetylornithine acetyltransferase and following the gene nomenclature of this yeast the locus will be named *ARG7* (*ECM40*) [30]. Four arg$^-$ mutants require arginine or citrulline, but not ornithine, suggesting that these mutants may be blocked in the ornithine to citrulline step catalyzed by ornithine carbamoyltransferase [31, 32]. According to the nomenclature used in *S. cerevisiae* and that previously used in *P. rhodozyma* [17], the locus will be named *ARG3*. Therefore, the mutant strains A2-92, A9-55, A10-68 and A18-89 may carry a mutated allele *arg3*$^-$ responsible for their arginine auxotrophy. On the other hand, five arg$^-$ and one albino-arg$^-$ mutants from wild-type CBS 7918 and two arg$^-$ mutants from strain S3-88 have an arginine deficient phenotype using arginine but not citrulline and are blocked in the citrulline to arginine step. Since citrulline is converted to argininosuccinate by argininosuccinate synthetase encoded by gene *ARG10* and then to arginine by argininosuccinate lyase encoded by gene *ARG4*, it is not possible to define which of these genes are affected in each strain. Additionally, strain A4-69 showed two auxotrophic requirements (arginine and methionine) but the genes involved in these reactions cannot be deduced from the feeding assays. The isolate S3-88A15-40 obtained from strain S3-88, showed a proline deficient phenotype, but cannot use ornithine, citrulline or arginine as supplements and therefore its phenotype does not involve mutations in the *PRO1* or *PRO2* genes, encoding for L-glutamate γ-phosphotransferase and L-glutamyl-P-reductase, respectively. This strain may be blocked in the pyrroline-5-carboxylate (P5C) to proline reaction of the proline biosynthesis pathway. Using *S. cerevisiae* as a model where this step is catalyzed by P5C reductase encoded by the *PRO3* gene, it is possible that this mutant carries a *pro3*$^-$ allele of this gene.

Long term storage of *X. dendrorhous* strains

Once a collection of mutants is obtained, it is necessary to store them for a long period of time in a viable form, maintaining their characteristics. In previous works we used standard protocols for yeast storage, as direct freezing at −70 °C of cell suspension in 15–20% glycerol without previous treatment. However no positive results were obtained specially with mutant strains. Therefore, two kinds of preservation methods applicable at the laboratory scale were tested. In the cryogenic methods, cells in 20% glycerol were submitted to different treatments before storage at −70 °C. Incubation at 4 °C for 2 h (CRYII) showed the highest cell viability of the wild type and mutant strains of *X. dendrorhous* after 65 months storage (Table 2). The percentages of cell survival obtained in the CRYII method were 2 to 38 times greater than the CRYIII method (incubation at −20 °C for 24 h), except for strain ATCC 24202 that showed very low survival in all CRY methods (0.003%). Incubation of cells with liquid nitrogen (CRYI method) showed the worst results with percentages of cell survival less than 0.002% for all preserved strains. Although the CRYII method showed acceptable percentages of survival, a high variation was observed among the strains preserved. These differences could be produced by the freezing process itself, because in all cryogenic methods and independently of the preserved strain, the principal decay in viable cell number was observed in the first ten days of storage, compared to the cellular titer before preservation (time 0), decaying slightly in the following months. An example is shown in Fig. 1 for ATCC 24202 and atx5 strains, which repre-

Table 2. Survival of wild-type and mutant strains of *X. dendrorhous* after 65 months of preservation by different methods.

Strain	Preservation Method CFU/ml (survival, %)							
	CRYI		CRYII		CRYIII		DGD	
CBS 7919	2×10^2	(0.001)	6×10^2	(0.008)	2×10^6	(0.3)	1×10^6	(1.4)
ATCC 24202	4×10^1	(0.00002)	6×10^3	(0.003)	5×10^3	(0.003)	9×10^6	(9.6)
NN385A13-21	3×10^3	(0.001)	5×10^6	(2)	2×10^5	(0.07)	5×10^6	(2.7)
atx5	1×10^3	(0.002)	3×10^6	(5.5)	1×10^6	(2.5)	1×10^6	(0.7)
atx6	3×10^3	(0.002)	3×10^5	(0.2)	5×10^5	(0.3)	4×10^6	(2.4)
NTG 252	2×10^3	(0.001)	4×10^6	(3)	3×10^5	(0.2)	4×10^6	(2.2)
NNatx5A17-11	3×10^3	(0.001)	2×10^6	(1)	2×10^5	(0.1)	3×10^6	(2.5)
NNatx6A10-6	2×10^2	(0.00008)	3×10^5	(0.1)	2×10^5	(0.07)	8×10^6	(2.1)

The percentage of survival was calculated in relation to the total cell number in each case. The values are the average of three independent experiments and errors associated to quantifications of yeast viability were in the range of 5 to 20% for all determinations.

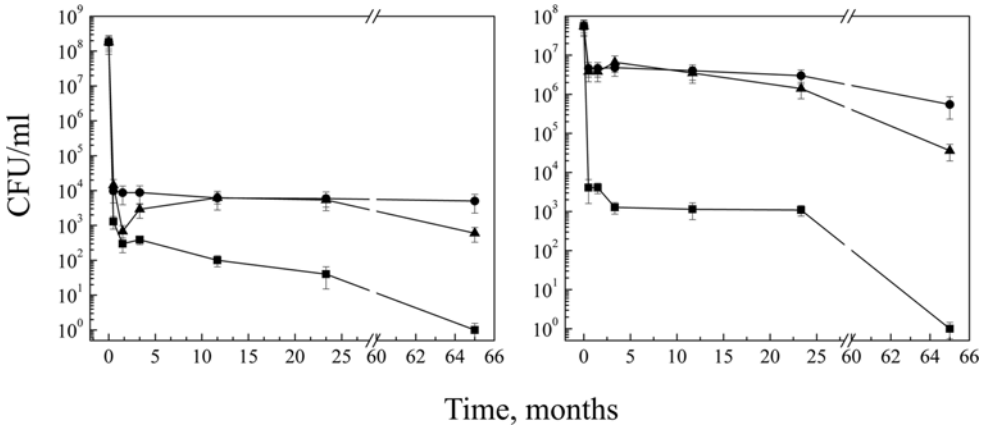

Figure 1. Storage of *X. dendrorhous* strains ATCC 24202 (A) and atx5 (B) by cryogenic methods. At each time the viable yeasts was determined as described in materials and methods. Data are the mean of CFU (±SE, $n = 3$) for yeast recovery after preservation by CRYI (■), CRYII (●) and CRYIII (▲) methods. Time 0 corresponds to yeast CFU before storage.

sents the worst and the best strains preserved by cryogenic methods, respectively.

When strains were preserved using the DGD method the percentages of cell survival were higher than those obtained using the cryogenic methods, varying from 1 to 10% (Table 2). This difference is more accentuated for strain ATCC 24202 that showed a decay of one and four orders of magnitude with the DGD and CRYII methods, respectively. Strains preserved by the DGD method for 27, 38 and 65 months were further characterized at macro and micro morphological levels, and in relation to their auxotrophic requirements (Table 1). All auxotrophic mutants checked maintained the original amino acid requirements. Mutants affected in the production of pigments maintain this phenotype unaltered and the wild type strain also continues to produce the characteristic orange/red colonies. Furthermore, mutants altered in the production of sexual structures maintained their ability to hyperproduce holobasidia (Fig. 2B) or the inability to develop these structures (Fig. 2C) after storage. Finally, no contamination with bacteria or fungus was observed in all the *X. dendrorhous* strains preserved by the DGD method or by the three CRY methods.

Discussion

Recessive mutants of *X. dendrorhous* as auxotroph mutants are rarely obtained, a characteristic reported previously [33] whereby it was necessary to increase the concentration of the mutagen NTG to 100 μg/ml with additional nystatin enrichment step [17]. In this work, the same experimental strategy was used to obtain auxotrophic mutants from strain CBS 7918 of *X. dendrorhous* and its derivative S3-88. An interesting result is that the frequency of auxotroph mutants obtained from this strain is 100 fold higher that reported in strain ATCC 24230 and its derivative mutants [17, 34]. The comparison of mutagenesis frequencies has been used as evidence in the determination of the ploidy levels in several organisms. These data suggest that the wild-type strain CBS 7918 could have a lower ploidy level (perhaps haploid), when compared to strain ATCC 24230. Furthermore, 42% of auxotrophs isolated from strain ATCC 24230 were ade$^-$, characteristic that was interpreted as areas of natural heterocigosity of the locus *ADE*, suggesting that this strain could be diploid [34]. In contrast, when the total number of auxotrophs obtained from CBS 7918 was analyzed, a restricted

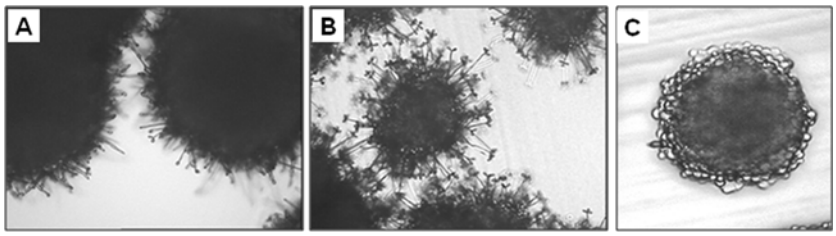

Figure 2. Macromorphology of *X. dendrorhous* strains preserved by the DGD method. The times of storage of wildtype (A), hol^{++} (B) and hol$^-$ (C) strains were 65, 38 and 27 months, respectively. Magnification is 10x for panel A and B, and 20x for panel C.

range of mutants was not observed (Retamales *et al.*, manuscript in preparation).

On average, the survival of *X. dendrorhous* strains preserved by the DGD method after 65 months of storage were one order of magnitude higher than those obtained by CRYII. The DGD method is based on storage of strains in anhydrous conditions and the results obtained indicate that *X. dendrorhous* strains assayed have more tolerance to desiccation (anhydrobiosis) than freezing. Anhydrobiosis is a natural occurring phenomenon and refers to cells with the ability to undergo almost absolute dehydration without loss of viability. Although the process is poorly understood, the central role of sugars as stabilizing compounds has been established [35–38]. We also observed stability of morphological characteristics, since *X. dendrorhous* wild-type strains did not show mutations and the mutant strains did not revert during the preservation period. Furthermore, the method provides easy storage because a high number of drops (60–80) could be kept in each tube, allowing the use of an individual drop without affecting the remaining ones. The most important parameter of this method is that samples must remain dehydrated. Parameters such as type and volume of vials, and number of drops stored in each vial are variable, since the method is versatile and adaptable to the requirements of each laboratory. We can conclude that DGD is a good method to store mutant strains of *X. dendrorhous* with the advantage that it is inexpensive and easy to perform, and allows a rapid growth of the yeast culture after preservation.

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