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Storage procedures for yeast preservation: phenotypic and genotypic evaluation

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Abstract - The aim of this study was to evaluate the morphological and biochemical characteristics of yeasts during storage. Six *Candida* spp. standard strains were stored in agarized medium with mineral oil in distilled water, frozen at -70 ºC and freeze dried. Strains were phenotypically characterised before being stored and then periodically for up to 18 months. Randomly Amplified Polymorphic DNA (RAPD) was carried out in time zero, 6, 12 and 18 months of storage. The viability of all samples was preserved except for the strain of *Candida dubliniensis* after 12 months of storage with mineral oil. No phenotypic alterations were observed in any of the methods employed. However, variations were observed in some phospholipase or proteinase activities. Changes in the RAPD patterns were not detected. These results seem to indicate that the maintenance methods tested were able to preserve the stability of the yeast phenotypic and genotypic characteristics.

Key words: yeast, preservation, storage, stability.

INTRODUCTION

Since microbiology is dependent on the availability of pure and stable cultures, the preservation of microorganisms is a very important task for laboratories in universities, hospitals, industries and other sectors. However, unless technical expertise is available to prevent it, microbial cultures may become contaminated, undergo phenotypic or genetic modifications or even die (Kirsop and Snell, 1984; McLellan and Day, 1995).

The interest in medical mycology and biotechnology has increased the demand for stable and correctly preserved yeast cultures (Kawamura *et al*., 1995). Because yeasts are unicellular organisms, they are generally easy to maintain and cultivate with simple nutritional and environmental requirements. Many techniques are available for microbial maintenance, and the main available techniques involve, for example, cell metabolism reduction using low temperature, reduction of available O_2 and/or dehydration of cells. The correct method to be employed in each laboratory must be determined according to parameters such as preservation time and the use of the proposed cultures (Spencer and Spencer, 1996).

Considering that studies on fungal biology, aetiology and epidemiology depend on stable strain characteristics, it is important to remember that during some types of storage, strains may undergo significant morphological, physiological, biochemical and even genetic alterations (Abadias *et al*., 2001; Santos *et al*., 2002; Lima *et al*., 2004). As a result, studies must be carried out in order to clarify the possible influence of the various storage methods available on yeast properties.

The aim of this study was to evaluate the viability of selected yeasts after maintenance by different preservation methods and the possible effects of these methods on the strains phenotypic and genotypic properties.

MATERIALS AND METHODS

Strains studied. Six standard yeast strains, four of medical and two of industrial importance, were selected for this study: *Candida parapsilosis* CBS 604^T (Centraalbureau voor Schimmelcultures), *Candida tropicalis* CBS 94^T, *Candida albicans* CBS 562^T, *Candida dubliniensis* CBS 7987^T, *Pichia jadinii* (*Candida utilis* CBS 5609^T) and *Kluyveromyces marxianus* [*Candida kefyr* IZ 1339 (Instituto Zimotécnico, Esalq-USP, Brazil)].

Preservation methods. The strains were submitted to five yeast preservation methods for up to 18 months as follows: periodic subculturing on agarized medium, agar slants with mineral oil, distilled water, freezing at -70 °C and freeze-drying (Jouan LP3 freeze-drier, Jouan Inc., Winchester, VA, US), according to Kirsop and Snell (1984). Each strain was preserved by each method in duplicate. Before being stored (time zero) the strains were submitted to standardised phenotypic tests applied to yeast identification, typing and characterisation and DNA was extracted for Randomly Amplified Polymorphic DNA (RAPD) analysis, as described below.

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Revival of samples. During the preservation period, samples were periodically reactivated and submitted to the selected phenotypic tests in triplicate. Cultures were incubated at 30 ºC for 48 h after being inoculated in Sabouraud Dextrose agar (SDA, Difco, Detroit, USA). Cultures that did not grow on SDA were inoculated in Brain Heart Infusion (BHI) broth (Difco). The revivals were carried out for up to 18 months at different time intervals according to the sample preservation method as follows: every month for strains conserved in agarized medium; every three months for those in distilled water and mineral oil; and every six months for frozen and freeze-dried cultures.

Evaluation of samples through phenotypic tests. The CHROMagar Candida® medium was used in order to evaluate possible contaminations, as well as the morphology and colour profile of the colonies. After that, representative colonies were sub-cultured on SDA and tested in relation to the micromorphology on Corn Meal agar (Difco) with 1% Tween 80. Germ tube formation, fermentation and assimilation of carbohydrates were also tested according to standard methods recommended by Kurtzman and Fell (1998). The assimilation of 10 carbohydrates (glucose, sucrose, galactose, raffinose, D-xylose, trehalose, maltose, lactose, melibiose and D-mannitol) was tested. Glucose, sucrose, galactose, maltose and lactose fermentations were also examined. The evaluation of the ability of strains to grow at 45 ºC was tested by streaking the culture over the surface of a SDA agar plate and incubating at 45 ºC for 48 h, as previously described (Mariano *et al*., 2003). Phospholipase and proteinase activities were determined by the plate method previously described (Price *et al*., 1982; Ruchel *et al*., 1982). After incubation for 4 and 7 days, respectively, the diameter of the colonies and the precipitation zones were measured. The enzyme activity (*P* value) was determined according to Price *et al*. (1982).

RAPD analysis. The RAPD analysis was carried out with DNA obtained from samples before conservation and every six months after being stored with the five different methods tested. Template DNA for RAPD analysis was prepared from yeast cells grown in 2 ml of YEPD broth (yeast extract 1%, peptone 1% and dextrose 2%) for 18 h at 30 ºC until a density of 2×10^8 cells/ml is achieved. DNA was extracted using a rapid, small-scale isolation protocol previously described (Mardegan *et al*., 2006). The oligonucleotide primers AP-3 – 5′TCACGATGCA3′ (Sanz *et al*., 1998); M-2 – 5′CTTGATTGCC3′ (Mello *et al*., 1998); B-14 – 5′GATCAAGTCC3′ (Bauer *et al*., 1993); RP4-2 – 5′CACATGCTTC3′ (Lehmann *et al*., 1992); R108 – 5′GTATTGCCCT3′ (Novak *et al*., 2004); OPA-02 – 5′TGCCGAGCTG3′ and OPA-09 – 5′GGGTAACGCC3′ (Pinto *et al*., 2004) were tested and those that produced the best band patterns were selected for the RAPD comparative assays. The RAPD reaction was performed according to Sansinforiano *et al*. (2001). RAPD profiles were defined with visual analysis, comparing the band patterns derived from the DNA strains extracted before sample storage with the patterns obtained after conservation using the different preservation methods.

RESULTS AND DISCUSSION

All five preservation methods tested maintained the samples viability during the analysed period, with the sole exception of *C. dubliniensis* (CBS 7987^T) that lost viability in mineral oil after 12 months of storage. Additional tests could reveal if the low survival rate is strain- or species-specific (Mendes da Silva *et al*., 1994).

In fact, some authors have documented high survival rates (89 to 100%) for up to 12 years (Odds, 1991; Qiangqiang *et al*., 1998) using simple storage methods such distilled water. Strain freezing and freeze-drying are very useful for long-term storage (20 years), and are commonly employed for the preservation of a variety of microorganisms, including yeast and microorganisms with special requirements (Kawamura *et al*., 1995; Qiangqiang *et al*., 1998; Crespo *et al*., 2000).

The present study also shows that the main phenotypic characteristics of the samples were preserved after storage using all methods described. The colony profiles in CHROMagar Candida® medium, the germ tube formation by *C. albicans* and *C. dubliniensis* and the growth ability at 45 ºC remained unchanged after storage in any of the conservation methods.

The occurrence of phenotypic modifications in stored microorganisms has been reported in literature (Abadias *et al*., 2001; Santos *et al*., 2002; Girão *et al*., 2004; Lima *et al*., 2004). Especially when they are maintained on agarized medium, the risks of morphological and/or physiological variations increase with the cultures age, where accumulated metabolism products may stimulate mutagenesis (Mendes da Silva *et al*., 1994). In spite of that, Ashcar *et al*. (1988) found that among the freeze-dried yeast cultures recovered after 34 years of storage, all of them conserved their original morphologic and physiologic characteristics. In addition, that study demonstrated that *C. albicans*, *Cryptococcus neoformans* and *Sporothrix schenckii* cultures conserved their patogenicity, verified through experimental inoculations.

Although the majority of the phenotypical tests have not shown changed results, the chlamydoconidia production by *C. albicans* and *C. dubliniensis* and the assimilation of certain carbohydrate became slower during storage, with late positive results (after 96 hours of incubation), in opposite to the fast positive reactivity (48 hours of incubation) in most samples. Such alterations occurred in different times in samples from different conservation methods and were not related to changes in the characteristics of the strains. In order to prevent such slow activities, the reactivation of the strains with two or three subcultures before tests and daily readings up to 7 days of incubation is recommended.

In this study, the proteinase and phospholipase production was less stable. Variability in the expression of these properties was observed in some strains in repeated tests during the study for one or more conservation methods (Tables 1 and 2). No correlation was observed regarding a specific conservation time period or yeast preservation method employed. *Candida parapsilosis* presented the most variability in all storage conditions for the proteinase test (17 changed results). Regarding phospholipase, the largest variability happened with *C. albicans* that presented 5 tests with different results. On the other hand, the enzymatic activity of *C. dubliniensis* and *Pichia jadinii* did

not change during the study for any class of enzymes. Proteinase and phospholipase production patterns are variable between different species. Other authors have mentioned constant phospholipase activity in individual isolates although a wide variation was found between different samples (Samaranayake *et al*., 1984; Williamson *et al*., 1986; Candido *et al*., 2000). Some works have shown that the enzymatic activity may change in function of factors such as the origin site of strains, the presence of lesions, the patient's immunological condition and test conditions such as pH variations and the concentration of sugars or nutrients in the medium (Price *et al*., 1982; Samaranayake *et al*., 1984). In addition, since these enzymes are virulence factors of *Candida* spp. that assist the organisms to invade and colonise host tissues, the long-term laboratory maintenance could influence negatively its expression, once the strain does not need to cause tissue damage.

Regarding the genetic characteristics, the RAPD patterns were not different in the samples before and after storage with the methods employed. In addition, multiple DNA extractions from the same isolate in a specific period result in the same RAPD patterns, suggesting the absence of genetic variations. Figure 1 shows the RAPD pattern for *C. dubliniensis* obtained with DNA from independent extractions.

A small number of studies have demonstrated the application of RAPD technique for microevolution detections. Our study seems to be the first one that attempts to evidence phenotypic and genotypic variations using RAPD in yeast samples preserved at different conditions. Phenotypic changes may be due to genetic modification. Genomic variations may be observed in strains with altered colony morphology, or other phenotypic changes (Rustchenko-Bulgac and Howard, 1993; Pesti *et al*., 2001; Novak *et al*., 2004). However, stable alterations in the morphological or biochemical aspects of the samples tested that could suggest genetic variations were not observed. Punctual mutations as base substitution are not easy to detect using most of the techniques, including RAPD, considering the random characteristic of the method.

Considering the storage methods applied in this research, all of them were capable of maintaining the samples characteristics during the period analysed. According to these results, it is possible concluding that retrospective studies, involving morphological and biochemical analysis, and even RAPD analyses could be applied in samples stocked using the five maintenance methods, observing the standardised conditions. Phenotypical characteristics related with essential functions and cell survival such as sugar assimilation and temperature resistance seem to be more

TABLE 1 - Yeasts with altered proteinase production in different tests (41) during 18 months of storage

Strain	Method of storage	Time of storage (months)	Initial enzimatic activity - T0*	Changed result after storage**
Candida parapsilosis	Subculturing	1	Negative	High activity
CBS 602	Subculturing	2		High activity
	Subculturing	3		High activity
	Subculturing	4		High activity
	Subculturing	7		High activity
	Subculturing	8		High activity
	Subculturing	9		High activity
	Subculturing	10		High activity
	Subculturing	14		High activity
	Distilled Water	9		High activity
	Distilled Water	12		High activity
	Distilled Water	18		High activity
	Mineral Oil	3		High activity
	Mineral Oil	9		High activity
	Mineral Oil	12		High activity
	Freezing	6		High activity
	Freeze-drying	6		High activity
Candida tropicalis	Subculturing	3	High	Moderate activity
CBS 94	Subculturing	5		Moderate activity
Kluyveromyces marxianus	Subculturing	5	High	Negative
IZ 1339	Subculturing	11		Negative
	Distilled Water	6		Negative
	Mineral oil	6		Negative
	Freezing	12		Negative
	Freezing	18		Negative
	Freeze-drying	18		Negative

*T0 – time zero of storage. ** Unchanged result after storage for *Candida albicans* CBS 562^T (high activity), *Candida dubliniensis* CBS 7987^T (high activity) and *Pichia jadinii* CBS 5609^T (negative).

TABLE 2 - Yeasts with altered phospholipase production in different tests (41) during 18 months of storage

Strain	Method of storage	Time of storage (months)	Initial enzimatic activity - T0	Changed result after storage*
Candida parapsilosis	Subculturing	4	Negative	Moderate activity
CBS 602	Subculturing	5		Moderate activity
Candida tropicalis	Subculturing	1	Negative	Moderate activity
CBS 94	Subculturing	2		Moderate activity
	Subculturing	4		Moderate activity
	Subculturing	5		Moderate activity
Candida albicans	Subculturing	1	High	Negative
CBS 562	Subculturing	2		Moderate activity
	Subculturing	6		Moderate activity
	Mineral oil	6		Moderate activity
	Freeze-drying	6		Moderate activity

T0 – time zero of storage.

* Unchanged result (negative activity) after storage for *Candida dubliniensis* CBS 7987^T, *Pichia jadinii* CBS 5609^T and *Kluyveromyces marxianus* IZ 1339.

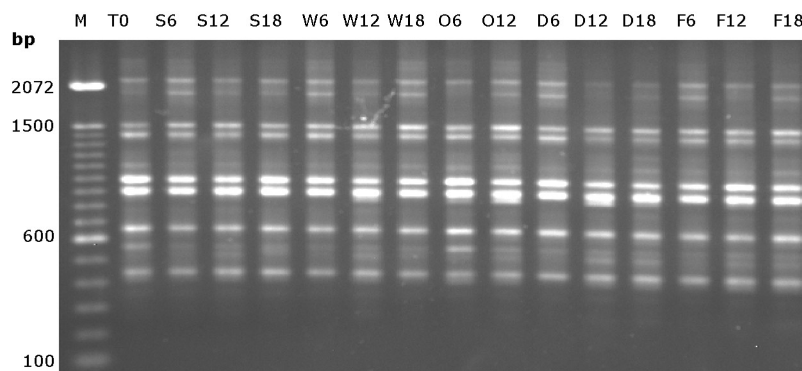

FIG. 1 - RAPD electrophoretic pattern of *Candida dubliniensis* (CBS 7987^T), with primer OPA-09, before and after the laboratorial maintenance through five different methods. M: 100 bp (DNA Ladder, Pharmacya Biotech), T0: time zero of storage, S: subculturing, W: distilled water, O: mineral oil, D: freeze-drying, F: freeze. Numbers 6, 12 and 18 indicate the storage time in months.

stable than those involved with specific functions as proteolytic enzyme production and other virulence factors.

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