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Evaluation of viability and stability of pathogenic mould and yeast species by using three different maintenance methods over a 12-year period

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Objectives

Maintaining and preserving fungal cultures are essential elements of systematics and biodiversity studies. Because fungi are such a diverse group, several methods of cultivation and preservation are required to ensure the viability and morphological, physiological, and genetic integrity of the cultures over time.

The collection of pathogenic fungi stored at the Mycology Reference Laboratory, Public Health Institute of Turkey (MRL-PHIT), Ankara, Turkey, contains over 1500 strains of fungi with medical relevance.

This study summarizes the results of attempts to revive a variety of mold and yeast isolates stored between 1 and 12 years using the three techniques available at the MRL-PHIT.

Methods

The list of fungal strains used in the study is provided in Table 1. Agar slants consisting of two screw-cap glass test tubes (16 x 9 x 125 mm) and one screw-cap, sterile, 5-ml polypropylene test tube (Cryo.s™ Cryogenic Storage Vials, Greiner Bio-One GmbH, Frickenhausen, Germany) containing potato dextrose agar (PDA, Oxoid Ltd, Basingstoke, UK) were inoculated with pure and fresh cultures of 567 yeast or yeast-like organisms and 619 mold isolates. The media was prepared according to manufacturer's recommendations, sterilized, and dispensed in 5 ml aliquots into the screw-cap glass and polypropylene test tubes. These media were used to preserve the strains by the following methods.

Water Culture Technique: Cultures inoculated into slants of PDA (Oxoid) in screw-cap glass test tubes were incubated at 25 °C for 2 weeks, after which 6–7 ml of sterile distilled water was added onto each culture. The spores and fragments of hyphae were removed by lightly scraping the aerial growth with the same pipette, and the resultant suspension was collected and transferred to a sterile screw-cap vial. The cap of the vial was tightened to prevent water evaporation, and sterile distilled water was added regularly if any evaporation was observed. The labeled vials were then stored at 25 °C on laboratory shelves. To check viability, approximately 0.3 ml of suspension from the vials was transferred under aseptic conditions either onto Sabouraud dextrose agar (SDA, Oxoid) or PDA slants. The cultures were then incubated at 25 °C for 3 weeks and observed regularly for growth. Those cultures that displayed no growth by the end of 3 weeks were retested with the same method. If no growth was detected after the second subculture, the isolates were recorded as not viable.

Freezing Technique: The fungal culture grown on a slant of PDA was cultured in a screw-cap polypropylene test tube and incubated at 25 °C for 2 weeks. The caps were tightly closed and placed into a freezer (−80 °C). To determine viability, the tube was removed from the freezer, and a small portion of the colony was chipped from the frozen PDA slant and inoculated onto the surface of PDA in a test tube. Frozen cultures were not allowed to thaw because of significantly diminished viability rates. The cultures were then immediately returned to the freezer. The fungal subculture was incubated at 25 °C for at least 3 weeks. If the viability and colony characteristics were consistent with the formerly known identification, the culture was recorded as viable. If cultures did not grow initially upon subculturing or if the colony characteristics were not consistent with the isolate's original identification, the fungal culture was then subcultured a second time using the method described above. The isolate was accepted as non-viable if no growth occurred after the second subculture attempt.

Oil Overlay Technique: Heavy grade mineral oil was sterilized by autoclaving for 15 min at 121 °C and 15 psi pressure in half-filled 250-ml flasks. Fungal cultures were incubated at 25 ° C for 2 weeks and grown on PDA screw-cap glass test tube slants. The fungal cultures were then covered with mineral oil up to 1 cm above the top of the slant. Care was taken such that the entire agar surface and fungal culture were completely submerged in the oil. The tubes were stored in an upright position at room temperature.

Table 1. Viabilities of isolates retrieved from storage in water, under mineral oil, and cryopreservation for 1–12 years (no. viable isolates/ no. tested isolates)

Species	Water stored	Mineral oil stored	Cryopreserved
Yeast			
Candida albicans	239/239	235/239	239/239
Candida dubliniensis	2/4	1/4	3/4
Candida glabrata	78/79	67/79	78/79
Candida guilliermondii	5/5	3/5	5/5
Candida kefyr	28/28	25/28	28/28
Candida krusei	27/27	21/27	27/27
Candida lipolytica	0/1	0/1	1/1
Candida lusitaniae	16/16	8/16	16/16
Candida parapsilosis	62/63	47/63	62/63
Candida pelliculosa	11/11	8/11	11/11
Candida tropicalis	70/71	68/71	71/71
Candida zeylanoides	3/3	2/3	3/3
Cryptococcus neoformans	3/3	2/3	3/3
Cryptococcus uzbekistanensis	1/1	1/1	1/1
Geotrichum candidum	1/1	1/1	1/1
Blastoschizomyces capitatus	1/1	0/1	1/1
Rhodotorula mucilaginosa	3/3	3/3	3/3
Saccharomyces cerevisiae	1/1	0/1	1/1
Trichosporon asahii	3/3	2/3	3/3
Trichosporon cutaneum	6/6	5/6	6/6
Trichosporon mucoides	1/1	1/1	1/1
Total yeast (%)	561/567 (98.9)	500/567 (88.2)	564/567 (99.5)
Molds			
Moniliaceous molds			
Aspergillus clavatus	2/2	1/2	2/2
Aspergillus flavus	1/1	1/1	1/1
Aspergillus fumigatus	11/14	10/14	14/14
Aspergillus nidulans	1/1	1/1	1/1
Aspergillus niger	5/6	6/6	6/6
Fusarium spp.	1/2	2/2	2/2
Mucor spp.	2/2	1/2	1/2
Rhizopus spp.	1/2	0/2	2/2
Scopulariopsis brevicaulis	2/3	1/3	3/3
Dematiaceous molds			
Alternaria spp.	2/2	1/2	1/2
Curvularia spp.	1/1	0/1	0/1
Fonsecaea pedrosoi	1/1	1/1	1/1
Dermatophytes			
Epidermophyton floccosum	2/6	1/6	4/6
Microsporum audouinii	0/1	0/1	1/1
Microsporum canis	16/19	10/19	19/19
Microsporum gypseum complex	0/1	0/1	1/1
Trichophyton interdigitale	144/151	133/151	146/151
Trichophyton rubrum	321/350	263/350	334/350
Trichophyton tonsurans	48/51	40/51	50/51
Trichophyton violaceum	1/3	0/3	2/3
Total molds (%)	562/619 (90.8)	472/619 (76.3)	591/619 (95.5)
General survival rate (%)	1123/1186 (94.7)	972/1186 (82.0)	1155/1186 (97.4)

The oil levels in the tubes were controlled regularly, and more oil was added when necessary. To revive a culture from mineral oil, a small amount of the fungal colony was inoculated on the appropriate media (PDA, SDA) after as much oil as possible had been drained. More than one subculture may be required after revival because the growth rate often remains slow because of adhering oil. Better results were achieved if the culture was inoculated at the midpoint of the agar slant, then allowing the oil to drain to the bottom as the culture grew upwards, away from the oil.

Stability: Stability was validated by determining the antifungal susceptibilities of eight random sample strains of five common yeasts [*Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. tropicalis* (n = 120)] stored by all three techniques. Amphotericin B, fluconazole, and itraconazole minimal inhibitory concentrations (MICs) were concluded following the Clinical and Laboratory Standards Institute (CLSI) guideline document M27-A2 before storage and 12 years after preservation. Extraction of fungal material and preparation for MALDI-TOF MS analysis were performed on a Bruker Autoflex III MALDI-TOF MS instrument (Bruker Daltonics, Bremen, Germany) as previously described. The acquired protein spectra were subsequently compared to a comprehensive library of reference spectra using the MALDI Biotyper software (database version 3.1.2.0) to obtain a list of the top matching identifications.

Results

Strain viabilities: Overall, 94.7 % of the water-stored fungal strains, 82.0 % of the under mineral oil-stored strains, and 97.4 % of the cryopreserved strains were viable, and their main morphological features corresponded to their original descriptions (Table 1).

The performance of each technique was superior for yeast isolates compared with mold isolates; the survival rates were 98.9 vs. 90.8 % for water, 88.2 vs. 76.3 % for under mineral oil, and 99.5 vs. 95.5 % for the cryopreservation method for yeast and mold isolates, respectively.

Stability: The stabilities of the yeast isolates were assessed by repeating the antifungal susceptibility test for a randomly selected group of *Candida isolates* (n = 120). The MICs for the isolates after storage were either the same as, or within three dilutions of, the MICs before storage.

Furthermore, a randomly selected group of fungal isolates (n = 365) representing the study population (dermatophytes, n = 115; yeasts, n = 250) that was stored by three different techniques was also further characterized by MALDI-TOF MS for species identification. The results obtained with MALDI-TOF MS displayed high agreement in species identification compared to the phenotypic methods used for the isolates tested (total, n = 365; dermatophytes, n = 115; yeasts, n = 250). However, the results obtained with MALDI-TOF MS and morphology based identification disagreed for two mold isolates (0.6 % of all isolates tested, 1.7 % of the dermatophytes tested).

Conclusion

After 12 years of preservation, the survival rates with the three different preservation techniques, i.e., in water, under mineral oil and by freezing, were assessed as 94.7, 82.0 and 97.4 %, respectively.

Viability was generally unrelated to the duration of storage. More stable and consistent growth was achieved after storage in water and freezing compared with mineral oil preservation.

Our results demonstrate that the procedure for maintaining fungal cultures in water is a simple and inexpensive method, next to cryopreservation, and that both can be reliably used for the long-term preservation of most fungal isolates.