



A new low-cost method for long-term preservation of filamentous fungi

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ABSTRACT

Preservation of fungi for commercial and research purposes for a long time, is a very tedious task. Cryopreservation and lyophilization have been extensively used as long-term preservation techniques. Here, we introduced a novel method for long preservation and fast revival of filamentous fungi using sterile cotton balls (CB). A 16 fungal genera and 47 species represented by 135 strains have been preserved on cotton balls moistened with Potato Dextrose Agar (PDA) and incubated at 25 ± 2 °C until giving sufficient growth, then preserved at 17–20 °C for 36 months. The revival of the fungal growth on PDA was monitored every 6 months. A 135 fungal strains have been revived by the CB method with an overall revival rate of 100% after two years of long-term preservation. While, 76 strains were revived after three years of preservation and the revival rate was 59%. The viability or the mycelial morphology was unaffected by the new method even after three years of preservation. It is an easy, cost-effective, and convenient method for preservation of filamentous fungi for long period without contamination risk. We believe that the CB method is a very convenient method for a cheap delivery of filamentous fungi between research institutes.

1. Introduction

According to the World Data Centre for Microorganisms (WDCM) there are about 585 culture collections recorded in 68 countries and these collections include a number of fungi reached 506333 species (Karaduman et al., 2012). The continuous isolation of new strains and the necessity to maintain such strains in order to improve research, systematics and biodiversity, biotechnology, functional, comparative and evolution genomics, bioprocesses and novel products studies, have stimulated many scientific and industrial research centers to maintain large collections of living microorganisms (Paul et al., 2015). The preservation of these microorganisms as pure cultures is a very important challenge in fungal identification in many culture collections worldwide (Hu et al., 2014).

Long term preservation of fungi without any changes in morphological or genetic information or physiological characters is the main purpose of culture collections (Karaduman et al., 2012; Smith and Ryan, 2012). So, several methods of cultivation and preservation are required to ensure the viability and morphological, physiological, pathogenic and genetic integrity of these cultures over time (Boundy-Mills et al., 2016). Short-term preservation involves maintenance of cultures for up to 1 year (Iqbal et al., 2017). Most fungal cultures can be maintained for that

period by serial transfer, which is simple, inexpensive, and widely used method. Although time-consuming and labor-intensive, a periodic transfer is a good option for small collections with cultures in constant use for short periods (less than 1 year) (Cui et al., 2018; Iqbal et al., 2017). The method also has several disadvantages, fungal cultures must be frequently transferred, checked for contamination by mites or other microorganisms and for drying. Also, the morphology and physiology of a cultured fungus may change over time (Fong et al., 2000). In particular, the ability to sporulate or to infect a host may be lost after repeated transfers (Ayala-Zermeño et al., 2017), doesn't prevent mutation and genetic variation (Homolka, 2013). Because of those disadvantages, the technique is generally inappropriate for long-term (more than 1 year) preservation of cultures. Short-term preservation of fungal cultures was difficult to maintain, due to transferring of these isolates at regular intervals there might be a risk of losing their virulence, pathogenicity, cultural and biological characteristics and they also require special equipment and continuous attention (Smith and Onions, 1983; Fong et al., 2000; Hu et al., 2014). Long term methods have been used for preservation of fungi include sterile water (Jones et al., 1991), under mineral oil (Perrin, 1979), sand (Smith and Ryan, 2012), under glycerol (Paul et al., 2015), silica gel (Perkins, 1962), and filter paper (Fong et al., 2000). These methods are also simple and cost-effective, but require

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Table 1
Fungal genera and species investigated for CB preservation method.

Genera	Species	Isolates No.	Revival after preservation period (months)					
			6	12	18	24	30	36
<i>Alternaria</i>	<i>A. alternata</i>	3	+	+	+	+	+	+
	<i>A. tenuissima</i>	3	+	+	+	+	+	+
<i>Aspergillus</i>	<i>A. clavatus</i>	3	+	+	+	+	+	-
	<i>A. flavipes</i>	3	+	+	+	+	+	-
	<i>A. flavus</i>	3	+	+	+	+	+	-
	<i>A. fumigatus</i>	3	+	+	+	+	+	+
	<i>A. niger</i>	3	+	+	+	+	+	+
	<i>A. ochraceus</i>	3	+	+	+	+	+	-
	<i>A. oryzae</i>	3	+	+	+	+	+	-
	<i>A. parasiticus</i>	3	+	+	+	+	+	-
	<i>A. tamarii</i>	3	+	+	+	+	+	-
	<i>A. terreus</i>	3	+	+	+	+	+	+
	<i>Chaetomium</i>	<i>C. globosum</i>	3	+	+	+	+	+
<i>Cladosporium</i>	<i>C. cladosporioides</i>	3	+	+	+	+	-	-
	<i>C. sphaerospermum</i>	3	+	+	+	+	-	-
<i>Curvularia</i>	<i>C. australiensis</i>	2	+	+	+	+	+	+
	<i>C. hawaiiensis</i>	2	+	+	+	+	+	+
	<i>C. lunata</i>	3	+	+	+	+	+	+
	<i>C. spicifera</i>	3	+	+	+	+	+	+
<i>Exserohilum</i>	<i>E. rostratum</i>	2	+	+	+	+	+	+
<i>Fusarium</i>	<i>F. equiseti</i>	3	+	+	+	+	+	+
	<i>F. oxysporum</i>	3	+	+	+	+	+	+
	<i>F. graminearum</i>	1	+	+	+	+	+	+
	<i>F. sambucinum</i>	3	+	+	+	+	+	+
	<i>F. semitectum</i>	3	+	+	+	+	+	+
	<i>F. solani</i>	3	+	+	+	+	+	+
	<i>F. verticillioides</i>	3	+	+	+	+	+	+
	<i>Lasioidiplodia</i>	<i>L. theobromae</i>	3	+	+	+	+	+
<i>Macrophomina</i>	<i>M. phaseolina</i>	3	+	+	+	+	+	+
<i>Neoscytalidium</i>	<i>N. dimidiatum</i>	5	+	+	+	+	+	+
<i>Nigrospora</i>	<i>N. oryzae</i>	3	+	+	+	+	+	+
<i>Penicillium</i>	<i>P. aurantiogriseum</i>	3	+	+	+	+	+	-
	<i>P. brevicompactum</i>	2	+	+	+	+	+	-
	<i>P. chrysogenum</i>	3	+	+	+	+	+	-
	<i>P. citrinum</i>	3	+	+	+	+	+	-
	<i>P. funiculosum</i>	3	+	+	+	+	+	-
	<i>P. griseofulvum</i>	3	+	+	+	+	+	-
	<i>P. oxalicum</i>	3	+	+	+	+	+	-
	<i>Stachybotrys</i>	<i>S. chartarum</i>	3	+	+	+	+	+
<i>S. virgatus</i>		1	+	+	+	+	+	+
<i>Stemphylium</i>	<i>S. botryosum</i>	3	+	+	+	+	+	+
	<i>S. vesicarium</i>	3	+	+	+	+	+	+
<i>Trichoderma</i>	<i>T. harzianum</i>	3	+	+	+	+	-	-
	<i>T. koningii</i>	3	+	+	+	+	-	-
	<i>T. longibrachiatum</i>	3	+	+	+	+	-	-
<i>Ulocladium</i>	<i>U. atrum</i>	3	+	+	+	+	+	+
	<i>U. botrytis</i>	3	+	+	+	+	+	+

regular subculture and fungal strains could be degenerate over the time of preservation (Hu et al., 2014). Cryopreservation, lyophilization, and preservation under liquid nitrogen are commonly used for long-term preservation of different groups of fungi (Ayala-Zermeño et al., 2017; Homolka, 2013; Linde et al., 2018; Hu et al., 2014). However, these methods are expensive, hazardous to handle, need special equipment and suitable for huge culture collection (Hu et al., 2014). As a result, various practical and inexpensive long-term methods were introduced for preserving fungal cultures (Eugenia et al., 2009). Therefore, the current method for long-term preservation of filamentous fungal species has been introduced. The aim of the present study is to find a suitable and cost-effective method for preservation of filamentous fungi. A low-cost substrate has been used for long-term preservation of several fungal species using cotton balls moistened with PDA medium. This work was conducted in the Culture Collection of the Assiut University Mycological Centre, Assiut University, Assiut Governorate, Egypt.

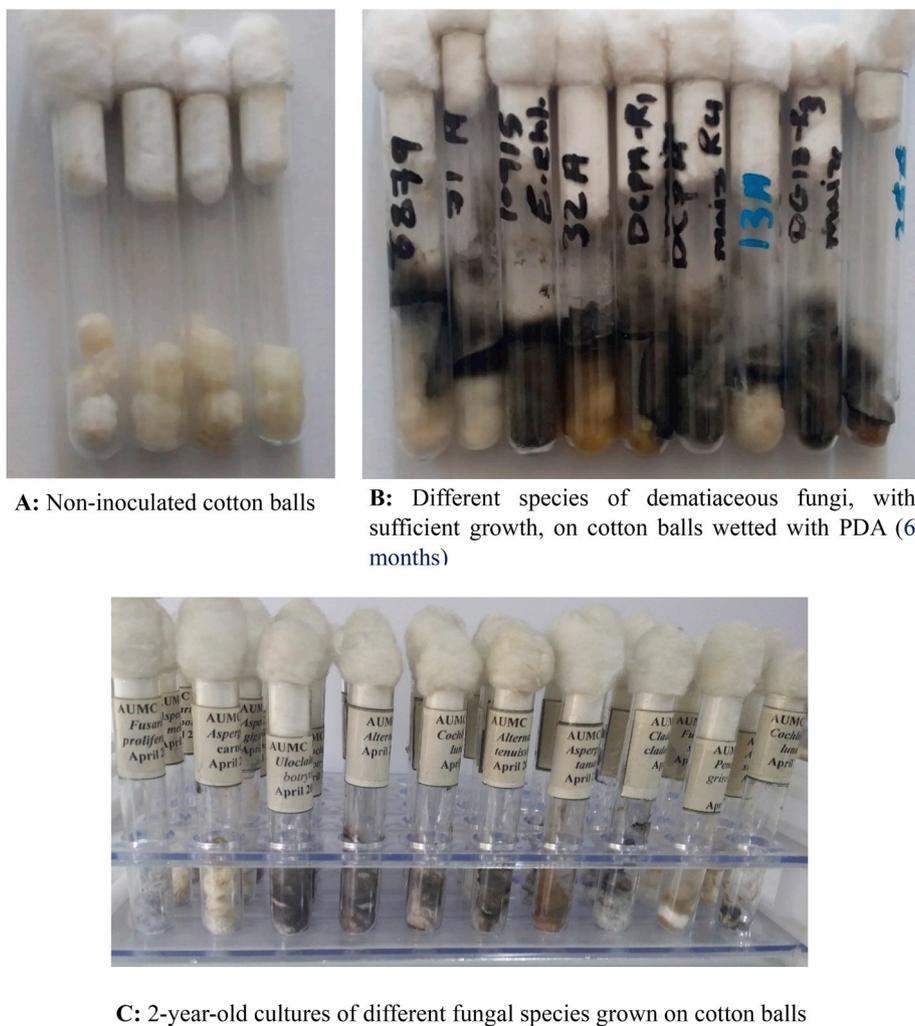
2. Materials and methods

2.1. Fungal species

All fungal strains were collected from Assiut University Mycological Centre (AUMC), Assiut University, Egypt. A total of 135 strains belonging to 16 genera and 47 species namely *Alternaria* (2 species), *Aspergillus* (10), *Chaetomium* (1), *Cladosporium* (2), *Curvularia* (4), *Exserohilum* (1), *Fusarium* (7), *Lasioidiplodia* (1), *Macrophomina* (1), *Neoscytalidium* (1), *Nigrospora* (1), *Penicillium* (7), *Stachybotrys* (2), *Stemphylium* (2), *Trichoderma* (3) and *Ulocladium* (2), were involved in the current study (Table 1).

2.2. Preparation of fungal cultures

Prior to preservation on cotton balls (CB), the fungal species were grown on Petri dishes (9 cm) containing Czapek's Dox agar with the composition of (g/L): sucrose, 30; sodium nitrate, 2; dipotassium hydrogen phosphate, 1; magnesium sulfate, 0.5; potassium chloride, 0.5; ferrous sulfate, 0.01, zinc sulfate, 0.01, copper sulfate, 0.005 and agar, 20 or PDA with the composition of (g/L): potatoes, infusion from 200 g;



A: Non-inoculated cotton balls

B: Different species of dematiaceous fungi, with sufficient growth, on cotton balls wetted with PDA (6 months)



C: 2-year-old cultures of different fungal species grown on cotton balls

Fig. 1. Showing A, Non-inoculated tubes; B, growth of fungal species on cotton balls; and C, 2-year-old fungal cultures maintained at 17 °C.



Fig. 2. Revival of some fungal strains after 24 months storage using CB method showing good growth of fungi. All the tested fungi were revived on PDA.

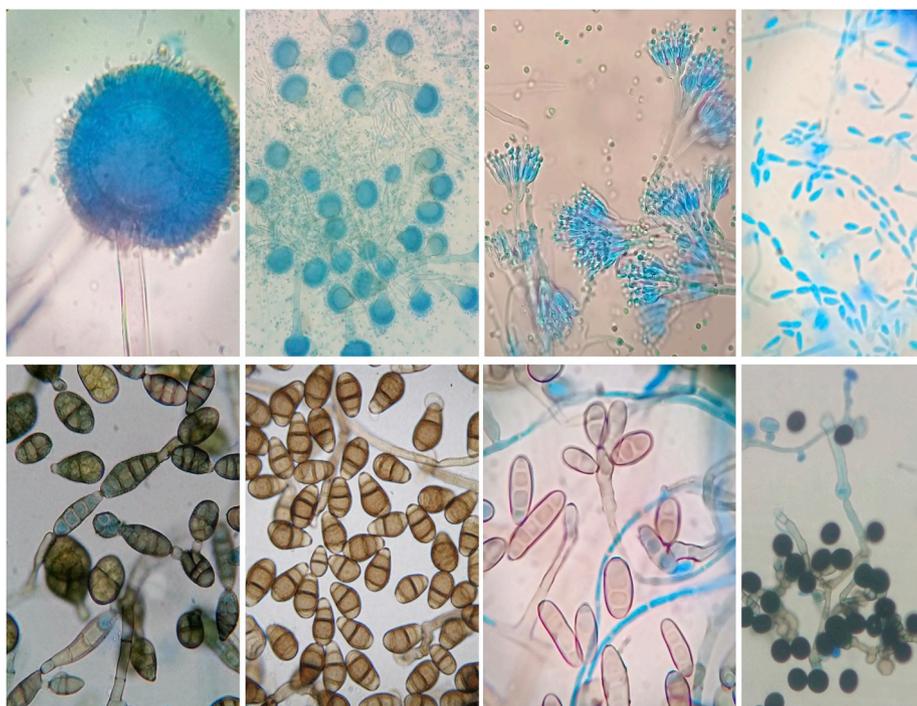


Fig. 3. Microscopic examination of some fungal strains after 36 months of storage using CB method.

dextrose, 20 and agar, 20; final pH 5.6 ± 0.2 . The inoculated plates were incubated at 28°C for 7 days. The grown fungi were used for inoculation of cotton balls.

2.3. Preservation conditions

In this study, we describe a new, simple and effective method for preservation of fungal species based on sterile cotton balls (CB) for long preservation and fast revival of filamentous fungi. A 3–5 balls (5 mm diameter) of natural cotton were placed in Wasserman tubes containing 2 ml of PDA. After autoclaving at 121°C for 20 min, the tubes were inoculated with the fungal strains to be maintained and then incubated at 28°C until a sufficient fungal growth was obtained as indicated in Fig (1). The grown fungal strains were preserved at a cooled place at 17°C – 20°C . Revival and bioavailability with the fungal growth were monitored in 6 months intervals for 36 months. The revival and recovery rate of fungal strains preserved by the CB was assessed by transferring inoculant from CB tubes to fresh PDA medium and incubated at 28°C and observed for growth daily.

3. Results

3.1. Viability test for fungal strains

The viability of fungal strains has been evaluated at zero time before they were submitting to the preservation test. After inoculation of the non-inoculated CB tubes (Fig. 1A) with the tested fungal strains, all tested fungi were able to grow as shown in Table 1 and Fig. 1B, C. Sixteen genera appertaining forty-seven species which represented by 135 isolates were subjected to the current method of preservation for 36 months and followed by testing the viability of the tested strains in 6 months interval. Melanized fungi; *Alternaria*, *Curvularia*, *Exserohilum*, *Lasiodiplodia*, *Macrophomina*, *Neoscytalidium*, *Nigrospora*, *Stachybotrys*, *Stemphylium*, *Ulocladium* in addition to *A. fumigatus*, *A. niger*, *A. terreus*, *C. globosum* and *Fusarium* spp. could survive for 36 months while the remaining *Aspergillus* species in addition to *Penicillium* species could survive for 30 months (Table 1). On the other hand, the maximum period of preservation for *Cladosporium* and *Trichoderma* species was 24 months (Table 1). There is no significant changes in the morphology of colony and mycelia after 36 months of preservation of tested strains by the CB method as shown in Fig. 2. The microscopic examination of some

Table 2

Estimated requirements of media, refrigerators, risk of contamination, revival percentage and cost for the preservation of a given culture collection contains 10000 fungal isolates.

Requirements	Preservation techniques					
	Frequent transfer		Oil overlay		Cotton balls	
	Quantity	Estimated Cost (\$)	Quantity	Estimated Cost (\$)	Quantity	Estimated Cost (\$)
Total No. of tubes	20000	500	20000	10000	20000	500
Total medium (Liter)	200	250	200	250	40	50
Oil (Liter)	–	–	200	500	–	–
Cotton (Kg)	80	400	–	–	10	50
Refrigerators (unit)	20	10000	20	10000	No need	0.00
Total cost		11150		20750		600
Cost/Fungus		1.115\$		2.075\$		0.06\$
Risk of contamination		Moderate to high		Moderate to high		Rare
Revival after 24 months (%)		75		75		100

preserved strains by the CB method is shown in Fig. 3. From the results of the microscopic examination before and after preservation, no changes have been observed after preservation by the CB method. These results suggest that the CB method is highly effective for maintenance of fungi and could be used for preservation of different fungal species. Also, this method is simple and did not require any complicated protocol.

3.2. Comparison between the cotton ball (CB), frequent transfer and oil overlay methods

The requirements and estimated cost for preservation, per a year, of a culture collection composed of 10000 fungal isolates, in a given culture collection, by the current method (CB), as well as the risk of contamination, were compared with the frequent transfer and the oil overlay methods, and they can be summarized in Table (2). It was indicated from Table (2) that the estimated cost by CB method for preservation of fungi is more economic compared to other frequent methods. Using CB is a possible alternative method, where the estimated cost for the preservation of fungi (0.06 \$/fungus) and estimated costs by oil overlay and frequent transfer is 2.075 and 1.115 \$/fungus, respectively. The risk of contamination was rare by CB method compared to other methods. Moreover, the percentage of revival and recovery of the culture after 24 months was 100% compared to 75% for the oil overlay and frequent transfer.

4. Discussion

Some fungi are characterized by the slow growth rate in culture media compared with bacteria, and in many times contamination by bacteria or other fungi may occur, which harms the preservation of these cultures. The most common methods of fungal presentation are submersion in sterile water, under mineral oil or glycerol, filter paper discs, woods, soils, and silica gel. These methods have been used for short- and long-term preservation of fungi. Cryopreservation in liquid nitrogen and lyophilization are also methods recommended for preservation of fungi by ATCC, but still expensive and require special equipment (Ayala-Zermeño et al., 2017; Cui et al., 2018; Hu et al., 2014). The existent techniques (lyophilization and cryopreservation) for the maintenance of the mycological culture collections are very difficult and costly, especially in huge culture collections (Iqbal et al., 2017; Linde et al., 2018). The preservation on slant culture is very simple, commonly used for preservation of microorganisms and inexpensive compared to other methods. However, it is time consuming, labor work, contamination by mites and in some cases death or drying of preserved cultures (Iqbal et al., 2017). Therefore, there is a necessity for the development of new methods for sufficient preservation of different fungi.

In the present study, 135 fungal strains related to 47 species were subjected to the current preservation method for 36 months using cotton balls, and the viability of the tested strains was observed in 6 months interval, with no alterations in the macroscopic and microscopic characteristics, demonstrating the efficiency of that method in the preservation of fungal culture viability. Previous studies showed that there is no single preservation method that can be employed for all fungi (Ryan et al., 2000; Smith and Ryan, 2012). Long term storage of fungi under mineral oil overlay may have led to physiological and morphological changes such as sporulation capability of fungi being reduced as well as a loss of virulence; as the fungi have covered by oil for a long time (da Silva et al., 1994). The preservation of fungal isolates in sterile water was unsuitable as most of the isolates died in 2–6 months during storage (Cui et al., 2018). Recently (Hu et al., 2014), used a new method for preservation of axenic fungal cultures using cellophane square sterile (CPS) method. This method is easy and simple. However, it requires to be placed in a sterile cryovial and frozen at -80°C . Other new methods require specific requirements, and, also expensive compared to our new method (Fong et al., 2000).

In the present investigation, melanized fungi such as *Alternaria*, *Curvularia*, *Exserohilum*, *Lasiodiplodia*, *Macrophomina*, *Neoscytalidium*, *Nigrospora*, *Stachybotrys*, *Stemphylium*, and *Ulocladium* in addition to *A. fumigatus*, *A. niger*, *A. terreus*, *C. globosum*, and *Fusarium* spp. could survive on the cotton balls for 36 months. The ability of some fungal species to survive on the cellulosic substrate (cotton balls) may be attributed to their abilities to produce cellulases, which enables these fungi to degrade the cotton fibers and extract the released glucose for their growth and metabolism.

5. Conclusions

This is the first report to use the cotton balls (CB) as a new method for preservation of filamentous fungi. We recommend the use of this method for preservation of different fungal strains because it is very simple, cost-effective, effective tool and convenient method for preservation of filamentous fungi without contamination risk, and requires very little refrigeration for the storage of the isolates for long period.

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