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Long Term Preservation of Commercial Important Fungi in Glycerol at 4°C

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ABSTRACT
Preservation of commercial and research important fungi for long time period is a very tedious job. Mostly cryopreservation technique in liquid nitrogen is used for long term preservation. In this work 15 different species of commercial and research important fungi are preserved at 4°C in different concentration of glycerol using two different methods (Slant culture and Slice cut method). In slant culture method at 50% of glycerol, 100 and 86.66% of fungi are viable upto 24 and 30 months of preservation, respectively. In slice cut culture method 100% of fungi are having regeneration capacity upto 24 months of preservation. This study help to preserve the fungi with easy and low cost for long term period at 4°C under refrigerator.

Key words: Concentration of glycerol, slant culture method, slice cut method, regeneration, preservation

INTRODUCTION
Many fungi are commercially used in the industries for the production of various products like; acetic acid, antibiotics, in food processing, bio-pest control, etc. So, their preservation for long time use is essential for industrial economy. The preservation of fungal culture is very tedious for long term period, usually this can be done by continuous subculture method and store in 4°C (Kitamoto *et al*., 2002; Yang and Rossignol, 1998). Fungal cultures and their medium are dried after some time and then the cultures are transfer to fresh medium at the interval of every six months. This method of preservation is time consuming, tedious and labour intensive job (Corbery and Le Tacon, 1997). Due to this sub-culturing, some types of drawback are also there, it changes the physiological and molecular characteristics due to continuous subculture and chances of rate of contamination is increases (Kitamoto *et al*., 2002). Now a day, mostly the fungal cultures are stored in liquid nitrogen by cryopreservation technique in -196°C for long term preservation (Hwang, 1966, 1968; Hwang and Howells, 1968; Corbery and Le Tacon, 1997; Espinel-Ingroff *et al*., 2004; Borman *et al*., 2006). There is a problem with this cryopreservation process, the cells and tissues of cultures are damaged due to ultra freezing shock and the cultures are not revive (Corbery and Le Tacon, 1997). Some cryoprotectants (glycerol, DMSO, ethylene glycol etc.) are used to solve this problem (Hwang, 1966, 1968; Hwang and Howells, 1968; Corbery and Le Tacon, 1997; Nagpal *et al*., 2012) but it does not protects the culture completely. The risk of damage to the user while handling of liquid nitrogen is a big problem. The availability, facility and cost of liquid nitrogen for small laboratory and labs situated in interior areas are also a big problem

(Stalpers *et al*., 1987; Ryan *et al*., 2000), so in this work we are tried to preserve the fungal culture in easy and simple way for long term period using traditional method of preservation in 4°C.

MATERIALS AND METHODS

Microorganisms: Total 15 different species of fungi are used for the long term preservation, most of these fungi are commonly present in the environment and commercially used in the industries for various purposes. Firstly the fungi cultures were grown in pre-poured Potato Dextrose Agar (PDA) medium plates at 25°C. Then after the sufficient growth, fresh cultures are used for further process. Composition for per liter of PDA was Potato 200 g, Dextrose-20 g, Agar 15 g.

Stock culture for preservation: Fungal colony was grown in two different methods; in first, the fungus of each colony was inoculated to autoclaved potato broth medium in Erlenmeyer flask. In second method the PDA medium was poured in transparent plastic tubes then autoclaved and prepared slants. Then fungal cultures are inoculated in the slant. The inoculated fungal cultures of both the methods were incubated for sufficient growth, after sufficient growth of mycelia and sporulation these two cultures are used as stock (Fig. 1).

Preparation for preservation: All the chemical and reagents are used in this study are of analytical grade from Merck specialities. Different concentration of glycerol was used as main preservative in this preservation technique. Broth cultures were cut into small-small pieces aseptically and deep into different tubes contains Distilled Water (DW), 15% glycerol, 50% glycerol and crude glycerol and slant cultures were submerged as same with distilled water, 15% glycerol, 50% glycerol and crude glycerol. Then both of the different cut and slant method cultures of fungi were sealed with cap lock. One set of different slant cultures was preserved without added any preservatives taken as control. The prepared stock cultures were then kept into refrigerator at 4°C (Fig. 1).

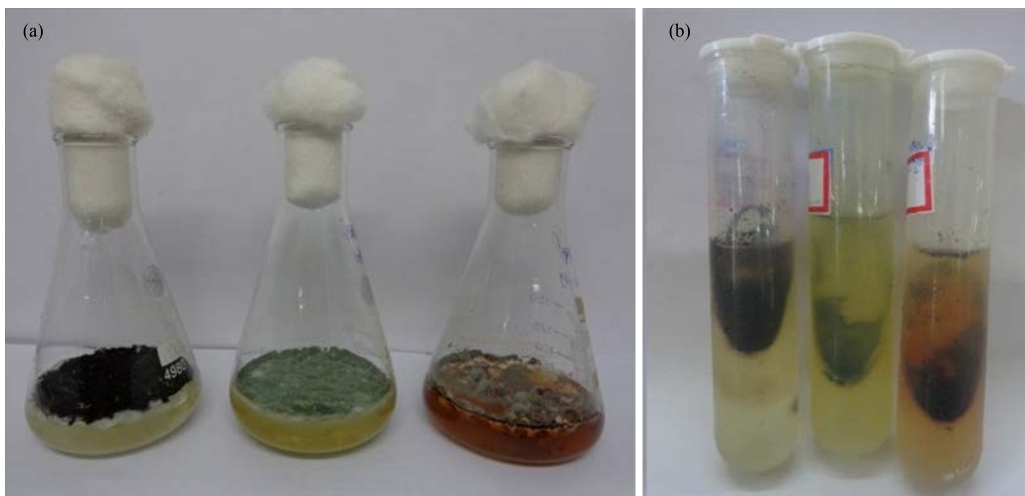

Fig. 1(a-b): (a) Stock and (b) Preserved cultures of fungi

Fig. 2: Revived cultures of fungi

Viability test: Viability test is very important step for the long term preservation, due to cold storage the cells and tissues of culture are damage and affect the regeneration capability, so it very important to check that the preserved cultures are viable are not at regular interval of time. So, fungal cultures are regularly tests for its regeneration capability in every six months, by using modified method of Espinel-Ingroff *et al*. (2004). Firstly, cultures are placed in room temperature for 10 min then thawing was done. After thawing, the cultures are picked from both the method (Cut and Slant) tube and inoculated into pre-poured PDA plates then incubated at 25°C for 10 days for growth. This viability tests are continuously performed in every 6 month upto 30 months (Fig. 2).

RESULTS

Preservation of fungal species is important for long time use in industries and research work, so it is essential to develop an easy and cheap technique for its preservation. Most of the previous work for the long term preservation of fungi was done through cryopreservation method using liquid nitrogen and commonly 5-10% of glycerol was used as cryoprotectant. In this study, first time we preserved industrial importance fungi under different concentration of glycerol for long term period at temperature of 4°C.

After every six months of re-culture, all the fungal cultures with control are viable and regenerate upto 12 months. After 12 months, control cultures was not regenerated due to drying of media and contamination was also found in some of the control cultures. Most of the fungi which was kept in the distilled water are viable only upto 18 months, accept *Aspergillus niger*, *A. flavus*, *Penicillium chrysogenum*, *P. rugulosum* and one *Rhizopus* sp. In 5% of glycerol all the fungi was viable and regenerate for 18 months in both the methods. In 15% of glycerol most of the fungi are viable upto 24 months, only *Aspergillus oryzae*, *A. niger* and *Penicillium rugulosum* was not recover in slant culture method and in slice cut method some more fungi was not regenerated. All the fungal cultures were viable and regenerated in both the methods upto 24 months in case of 50% glycerol. For 30 months, in 50% of glycerol all the fungi were regenerated in slant methods except *Aspergillus niger* and *A. fumigatus* and in slice cut method five cultures are not regenerated. In crude glycerol fungal cultures, most of the fungi are viable upto 24 months and for 30 months some of them are not viable in both of the methods. According to the above result it is clearly showed that 50% of glycerol was better for long term preservation upto 30 months in slant method and in slice cut method it is preserved for 24 months in low temperature of 4°C (Table 1).

Table 1: Fungal culture viability test of slant culture and slice cut culture method

Fungi	Control					DW					5 (%)					15 (%)					50 (%)					Crude				
(Month)	6	12	18	24	30	6	12	18	24	30	6	12	18	24	30	6	12	18	24	30	6	12	18	24	30	6	12	18	24	30
Slant culture																														
Aspergillus oryzae	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Aspergillus fumigatus	+	+	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-
Aspergillus flavus	+	+	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Aspergillus versicolor	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Aspergillus niger	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	-	-
Penicillium godlewskii	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Penicillium notatum	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Penicillium chrysogenum	+	+	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Penicillium rugulosum	+	+	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-
Penicillium janthinellum	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Rhizopus nigricans	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Rhizopus oligosporus	+	+	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Trichoderma viride	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Trichoderma reesei	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Fusarium verticillioides	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Slice cut culture																														
Aspergillus oryzae	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Aspergillus fumigatus	+	+	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
Aspergillus flavus	+	+	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
Aspergillus versicolor	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
Aspergillus niger	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Penicillium godlewskii	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
Penicillium notatum	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Penicillium chrysogenum	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Penicillium rugulosum	+	+	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	-	-
Penicillium janthinellum	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Rhizopus nigricans	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Rhizopus oligosporus	+	+	-	-	-	+	+	-	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
Trichoderma viride	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Trichoderma reesei	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
Fusarium verticillioides	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-

DW: Distilled water, (5, 15, 50%, Crude): Concentration of glycerol, +: Revived, -: Not revived

Table 2: Regeneration percentage of fungal culture

	Viability (%)	
Concentration of glycerol and months	Slant method	Slice cut method
Control		
6	100.00	100.00
12	100.00	100.00
18	13.33	13.33
24	0.00	0.00
30	0.00	0.00
DW		
6	100.00	100.00
12	100.00	100.00
18	66.66	73.33
24	0.00	0.00
30	0.00	0.00
5%		
6	100.00	100.00
12	100.00	100.00
18	100.00	100.00
24	0.00	0.00
30	0.00	0.00
15%		
6	100.00	100.00
12	100.00	100.00
18	100.00	100.00
24	80.00	66.66
30	0.00	0.00
50%		
6	100.00	100.00
12	100.00	100.00
18	100.00	100.00
24	100.00	100.00
30	86.66	66.66
Crude		
6	100.00	100.00
12	100.00	100.00
18	100.00	100.00
24	86.66	93.33
30	73.33	66.66

Regeneration percentage of fungal culture was also calculated to estimate the best preservation method and its concentration of preservatives. It is an important factor to determine the potential method viability of fungus. In control culture the regeneration capability was 100% only upto 12 months and for 18 months it decreases upto 13.33%, after 18 months the viability percentage of control fungal culture was zero. The maximum viability of 100% was found in 50% of glycerol upto 24 months and 86.66% upto 30 months in slant cultured method. In slice cut method, the maximum regeneration of fungal culture was determined 100% up to 24 months (Table 2).

DISCUSSION

In this study PDA medium was used for the culture of fungi, it was easy to prepared, having low cost and its ingredients are easily available, this medium is differential medium. All type of fungal cultures is sufficiently grown in short period of time in PDA medium. Most of the other mediums are synthetic medium, they are costly and tedious for preparing due to its large and costly chemical composition and the growth of fungi is also affected due to its complete synthetic properties. So in this work PDA medium was used for the growth of fungi.

Due to long time of preservation and frozen effect, fungal cultures are losing their regeneration properties and many of the fungi which is used for the production of different compounds are loss

their production capability. Glycerol plays an important role and acts as a cryoprotectant for fungi, it maintains regeneration activity and prevent from damage. So, different concentration of glycerol was used as cryoprotectant in this work for long term preservation of fungi at 4°C and other researchers are also used glycerol for preservation techniques but they applied it for cryopreservation. Yang and Rossignol (1998), Hwang (1966, 1968) and Lalaymia *et al*. (2014) are also suggested that glycerol is most appropriate preservative for the long term preservation of fungi in liquid nitrogen. Ryan *et al*. (2000) suggested the different key techniques and protectants which are suitable for sporulated fungal culture preservation. Smith and Thomas (1997) described the cooling process for the preservation of filamentous fungi, they preserve and test nine different strain of fungus regeneration capacity used glycerol as preservative. Nagpal *et al*. (2012) found that glycerol was effective for the preservation of fungi maximum upto 90 days at -70°C. Kitamoto *et al*. (2002) preserve the cutting dick fungal mycelia method in saw dust medium at -85°C and they also found glycerol was effective cryoprotectant for the most of macro-fungi up to 33 months.

CONCLUSION

Preservation and maintenance of fungi for long term period is a basic need for research and industrial purpose. Mostly cryopreservation technique is used for long term preservation but in this work for the first time different concentrations of glycerol was used as a preservative for long term preservation in low temperature 4°C under refrigerator. The maximum viability of 100% was found in 50% of glycerol upto 24 months and 86.66% upto 30 months in slant cultured method. Through this technique it was clearly showed that fungi were preserved up to 24-30 months without any contamination. This method of preservation is easy, low cost and also applicable in remote areas where they don't have cryopreservation facilities.

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