Preservation Technique of Filamentous Fungi Based on Inactive Metabolism at Indonesian Culture Collection (InaCC)

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Abstract. Preservation is the act of preserving to keep, maintain, and conserve microbial strains to ensure their optimal viability and genetic stability. A wide variety of preservation techniques are available for preserving filamentous fungi. Most of the metabolically inactive preservation, such as freezing, cryopreservation, lyophilization, or L-drying, is widely accepted for long-term preservation of filamentous fungi with minimum viability loss and genetic changes. This study determined the viability and effectiveness of freezing or cryopreservation method in a fungal assemblage from the Indonesian Culture Collection (InaCC). The viability and growth rate of 40 fungal strains from InaCC were tested after three years of storage in the electric deep freezer (-80ºC with 10% glycerol and 5% trehalose as cryoprotectant). The results showed that 95% of the observed strains could maintain their viability and growth rate after cryopreserved and storage for more than three years at -80ºC. However, 5% of them showed a low growth rate and loss of viability. It was concluded that the cryopreservation technique by freezing at -80ºC is suitable to maintain the viability of filamentous fungal cultures.

1. Introduction

Fungi are organisms with the second-highest biodiversity after insects. They are eukaryotic, heterotrophic, spore-producing organisms that absorb their nutrition. Fungi mostly reproduce sexually and asexually and usually have filamentous, branched, a somatic structure known as hyphae that typically surrounded by a cell wall [1]. According to [2], fungi are widely used in industries and commercial purposes such as bio-pest control, acetic acid, antibiotics, and food processing. Therefore, fungal preservation in a suitable condition is essential to underpin the basic and applied research as well as for priceless bioresources in ex-situ conservation.

Preservation is a preserving process to avoid deterioration and viability loss. It is intended to keep the genetic features and characters of microbial culture remain viable and stable until they are revived for future use [3,4]. A preservation intends to keep the low rate of microbial metabolism activity. Thus, their viability or growth can be maintained. It also maintains the microbe to recover or capable of re-growth with high survival and minimum character changes [5].

Short-term microbial storage is usually done by subculturing or transfer the cultures periodically into new fresh media. These techniques are time consuming and laborious. Some other simple techniques are useful for keeping the culture in short- or medium-period, but they are mostly unsuitable for long-term storage. The simple techniques include storage in mineral oil, liquid paraffin,

sterile soil, sterile water, porcelain beads, gelatin plates, and P_2O_5 in a vacuum. They require only simple and accessible equipment, which can be useful for institutions that do not yet have an advanced tool [6].

Fungal preservation methods based on inactive metabolisms such as freezing and lyophilization are widely implemented for preserving and storing fungi for a long period with a low mortality rate. Both methods decrease the fungal metabolic rate and induce dormancy with low mortality [7]. During normal metabolism, long-term storage of microbes accumulate metabolites products, which later could be toxic to the microbial cells and damaged them. As a result, it decreases the microbe viability and damages the isolate in the storage. Therefore, the microbe metabolisms should remain inactive during the preservation method [8].

Based on the previous description, this paper discusses the effectiveness of cryopreservation in preserving filamentous fungal cultures. The objectives of this study were to determine the viability and effectiveness of cryopreservation method for fungal strain assemblage from Indonesian Culture Collection (InaCC) after three-year storage in the electric deep freezer (80°C) with 10% (v/v) glycerol and 5% (g/v) trehalose as cryoprotectant.

2. Methods

This work was conducted at Indonesian Culture Collection (InaCC), Indonesian Institute of Sciences (LIPI), Cibinong Science Center, Cibinong, West Java, Indonesia), from 22 January up to 22 February 2018.

2.1. The Laboratory Instruments Preparation

The laboratory equipment and instruments which should be prepared were a toothpick and thermostable plastic straw. The sterile straw was prepared by cutting it into ± 4-5 cm, then placing them in a Petri dish. A similar method was applied to sterilize toothpicks. The dish was wrapped with used paper before sterilizing in an autoclave for 15 minutes at 121°C and 2 atm.

2.2. Medium Preparation

The potato dextrose agar (PDA) medium stock was weighed with an analytical scale for 39 g then put into a Beaker glass with 1 L distilled water. The suspension was placed on a hotplate and homogenized using a magnetic stirrer to completely dissolved the stock. The pH medium was adjusted to 7 (pH universal paper indicator). The medium was stored in aluminum sealed teapot and sterilized in an autoclave (15 minutes, 121°C, 2 atm). The medium then aseptically poured into the Petri dish in the LAF.

2.3. Cryoprotectant Preparation

The cryoprotectant was prepared based on [9] with slight modification using 10% (v/v) glycerol and 5% (g/v) trehalose. The preservation of media was made by measuring 20 mL of absolute glycerol stock and 180 mL of distilled water (10% glycerol) into the Beaker glass, then 10 g of 5% trehalose was added to the glass. The solution was homogenized with a stirrer on a hotplate. One ml of the solution was put into a cryotube then covered with a screw cap. The cryotubes were autoclaved for 15 minutes at 121°C and 2 atm.

2.4. Cryopreservation by Freezing Methods

The cryopreservation methods followed [9], in which the fungal strains were grown on PDA medium in the Petri dish. Fungal cultures on early stationary phase (5-7 days incubation at 27°C) were prepared [9]. The fungal culture of various phases growth representing the stationary, logarithm, and lag phase were cut using a sterilized plastic straw to form agar disc-shaped. As many as 8-10 of fungal discs were placed into the cryotube containing sterile 5% trehalose and 10% glycerol. The cryotubes were put into the cryo box and were kept in the refrigerator at 4°C for 6 hours-overnight, then moved into the electric deep freezer for long storage at -80°C.

2.5. Viability test

The viability and purity tests were done by using a modified method of [10]. The cultures were selected from the deep freezer (-80°C) and immediately placed in a water bath (37°C for 3 minutes) to thaw. The disc-shaped cultures in the cryotube were inoculated to PDA media in the Petri dish using a sterilized toothpick. They were incubated at room temperature for 5-10 days to grow. The tests were continuously performed after one, six up to 30 months of storage [10].

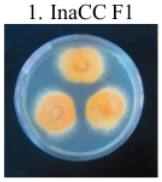
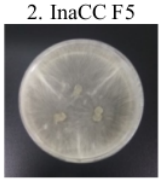
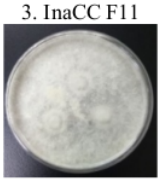
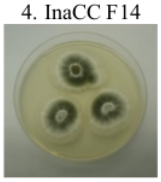
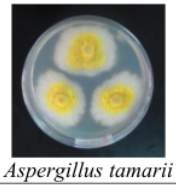
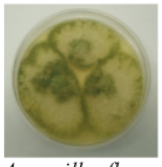
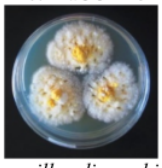
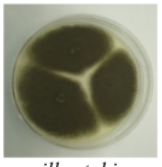
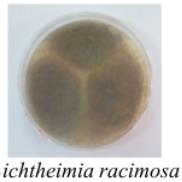
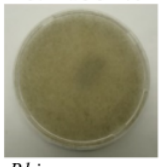
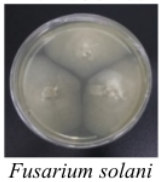
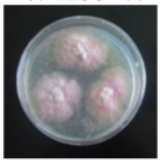
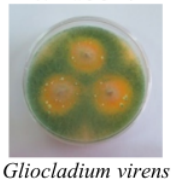
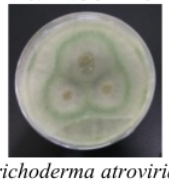
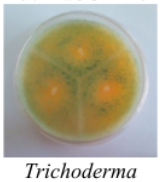
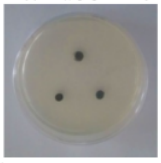
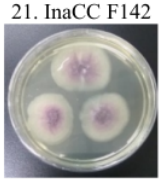
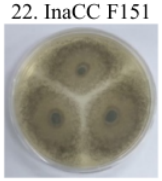
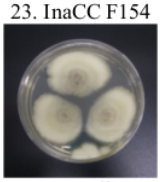
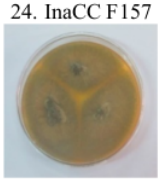
3. Results

Table 1 shows the growth rates and viability test of the fungi preserved for about three years. Two fungal strains, i.e., *Agaricus campestris* (InaCC F126) and *Xylaria* sp (InaCC F232), have not shown any growth indicating lost their viability. No growth (indicating by colony diameter size increase) were detected during the first 24 hours, but after 48 hours, they grow for 0.5 cm, and after 72 hours, their colony diameter reached 1.5 cm. The fungi have fully covered the Petri dish after 84 hours and 120 hours (colony diameter > 3 cm).

Table. 1. Accelerated viability tests and growth rates of 40 InaCC fungal strains grown in the disposable Petri dishes (Diameter 8 cm).

No	InaCC No	Funga Taxa	Viability Result			Growth rate /Colony diameter average (cm)					Notes
			Viability	Purity	Identity	24 h	48h	72h	84h	120h	
1	InaCC F1	*Monascus purpureus*	+	+	+	0	0	0,2	0,5	1	
2	InaCC F5	*Sclerotium rolfsii*	+	+	+	0	0,2	1	3	>3	
3	InaCC F11	*Ganoderma lucidum*	+	+	+	0	0	0	0,3	0,7	
4	InaCC F14	*Penicillium griseofulvum*	+	+	+	0	0	1	1.4	2	
5	InaCC F16	*Penicillium bilaiae*	+	+	+	0	0	0,5	1	1,5	
6	InaCC F22	*Aspergillus niger*	+	+	+	0	0	1	1,4	2,5	
7	InaCC F39	*Aspergillus tamarii*	+	+	+	0	0	0,8	1,2	2	
8	InaCC F43	*Aspergillus oryzae*	+	+	+	0	0	1	1,5	2,5	
9	InaCC F44	*Aspergillus flavus*	+	+	+	0	0	1	1,7	2,5	
10	InaCC F48	*Aspergillus dimorphicus*	+	+	+	0	0	0,5	1	2	
11	InaCC F61	*Aspergillus tubingensis*	+	+	+	0	0	1	1,7	2,5	
12	InaCC F71	*Lichtheimia ramosa*	+	+	+	0	0	1	2,5	>3	
13	InaCC F75	*Rhizopus oryzae*	+	+	+	0	0	1	3	>3	
14	InaCC F76	*Fusarium solani*	+	+	+	0	0	0,6	1	1,5	
15	InaCC F78	*Fusarium oxysporum*	+	+	+	0	0	0.8	1,2	2	
16	InaCC F94	*Gliocladium virens*	+	+	+	0	0,2	1	2,5	>3	
17	InaCC F100	*Pleurotus cystidiosus*	+	+	+	0	0	0	0,2	0,5	
18	InaCC F113	*Trichoderma atroviride*	+	+	+	0	0	0,3	1	3	
19	InaCC F115	*Trichoderma harzianum*	+	+	+	0	0,2	1	3	>3	
20	InaCC F126	*Agaricus campestris*									no growth
21	InaCC F142	*Fusarium oxysporum*	+	+	+	0	0	0,8	1,2	2,5	
22	InaCC F151	*Syncephalastrum racemosum*	+	+	+	0	0	1	3	>3	
23	InaCC F154	*Eupenicillium javanicum*	+	+	+	0	0	0,2	0,8	1,5	
24	InaCC F157	*Chaetomium globosum*	+	+	+	0	0	0,2	0,5	1	
25	InaCC F167	*Cercospora acalyphae*	+	+	+	0	0	0	0,2	0,5	
26	InaCC F187	*Cercospora helianthicola*	+	+	+	0	0	0	0,2	0,6	
27	InaCC F200	*Trametes versicolor*	+	+	+	0	0	0,5	1	2,5	
28	InaCC F206	*Phanerochaete chrysosporium*	+	+	+	0	0	1	2,5	>3	
29	InaCC F209	*Pleurotus ostreatus*	+	+	+	0	0	0	0,5	1	
30	InaCC F212	*Curvularia lunata*	+	+	+	0	0	0,4	0,8	1,5	
31	InaCC F215	*Pleurotus sajor-caju*	+	+	+	0	0	0,4	1	2	
32	InaCC F216	*Pleurotus ostreatus*	+	+	+	0	0	0,8	1,5	2,5	
33	InaCC F225	*Rhizopus oligosporus*	+	+	+	0	0	1	3	>3	
34	InaCC F226	*Neurospora intermedia*	+	+	+	0	0,5	1,5	>3	>3	
35	InaCC F232	*Xylaria sp.*									no growth
36	InaCC F251	*Colletotrichum acutatum*	+	+	+	0	0	0,5	1	2	
37	InaCC F287	*Phomopsis helianthi*	+	+	+	0	0	0,8	1,2	2,5	
38	InaCC F798	*Curvularia geniculata*	+	+	+	0	0	0,5	1	1,8	
39	InaCC F807	*Phylosticta capitalensis*	+	+	+	0	0	0,2	1	1,5	
40	InaCC F808	*Neopestalotiopsis asiatica*	+	+	+	0	0	1	1,5	2,5	

Figure 1. shows non-growing InaCC F126 and InaCC F232 due to loss of their viability, whereas others demonstrated good viability. The best growth was shown by isolates InaCC F226 that fully covered the Petri dish in the 72 hours.

1. InaCC F1  *Monascus Purpureus*	2. InaCC F5  *Sclerotium rolfsii*	3. InaCC F11  *Ganoderma lucidum*	4. InaCC F14  *Penicillium griseofulvum*
5. InaCC F16  *Penicillium bilaiae*	6. InaCC F22  *Aspergillus niger*	7. InaCC F39  *Aspergillus tamarii*	8. InaCC F43  *Aspergillus oryzae*
9. InaCC F44  *Aspergillus flavus*	10. InaCC F48  *Aspergillus dimorphicus*	11. InaCC F61  *Aspergillus tubingensis*	12. InaCC F71  *Lichtheimia racimosa*
13. InaCC F75  *Rhizopus oryzae*	14. InaCC F76  *Fusarium solani*	15. InaCC F78  *Fusarium oxysporum*	16. InaCC F94  *Gliocladium virens*
17. InaCC F100  *Pleurotus cystidiosus*	18. InaCC F113  *Trichoderma atroviride*	19. InaCC F115  *Trichoderma harzianum*	20. InaCC F126  *Agaricus campestris*
21. InaCC F142  *Fusarium oxysporum*	22. InaCC F151  *Syncephalastrum racemosum*	23. InaCC F154  *Eupenicillium javanicum*	24. InaCC F157  *Chaetomium globosum*

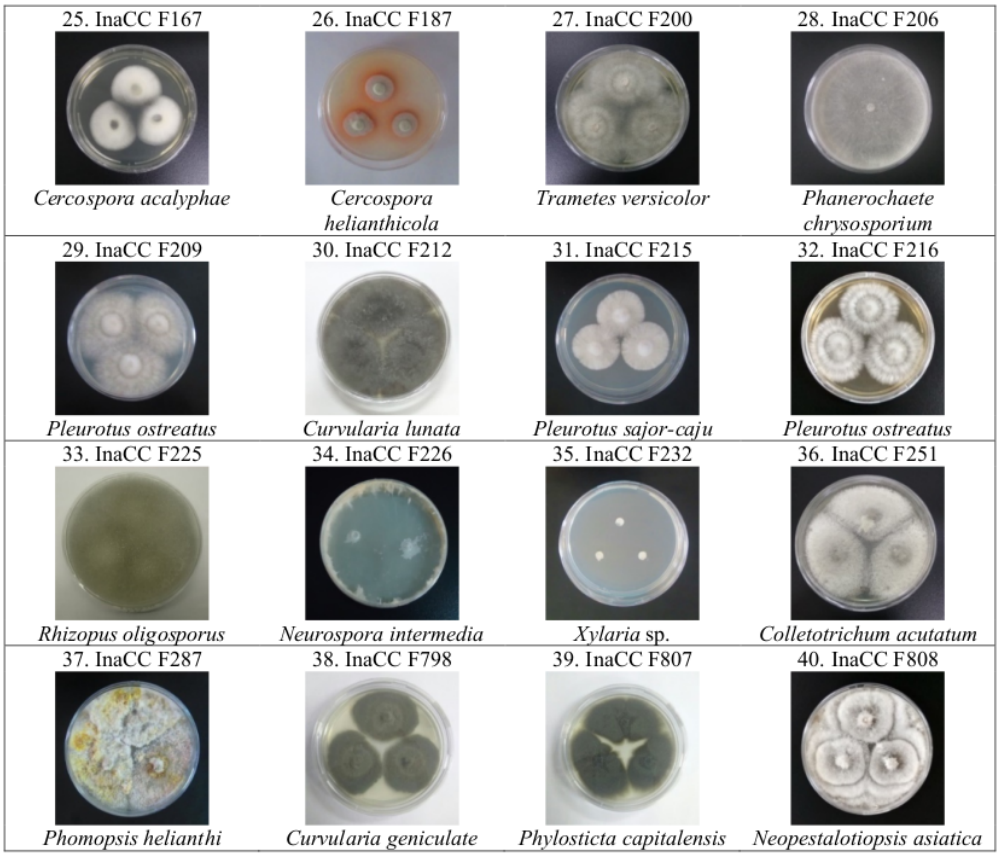


Figure 1. InaCC fungal strains for cryopreservation and viability test

4. Discussion

Two strains of fungi, InaCC F126 and InaCC F232, have lost their viability, but InaCC F226 (Neurospora intermedia) demonstrated the fastest growth rate. Neurospora is a member of Ascomycetes phylum, which useful for a model in the genetic study and widely used for commercial values [11]. Neurospora has known as the fastest-growing filamentous fungal species [12]. Its growth rate has reached approximately 10 cm per day or 5 mm per hour [12,13]. The growth rates of Neurospora hyphal tips are 19.1-42.5 µm per minute at 32°C, and the suitable pH for vegetative growth ranges from 5 to 7 (optimum of pH 5.5) [11]. It is also stated that the cryopreservation is the most suitable long-term preservation technique for Ascomycetes phylum by [14]. The cryopreservation seems to be the best preservation technique available for filamentous fungi [15].

The factors causing loss of viability in fungi were repeated thawing and freezing. Repeated freezing and thawing significantly reduce the culture viability [16]. Therefore, some factors should be observed before doing the cryopreservation method in which the microbes must be a pure culture, and the storage environment must be maintained [8]. Many factors including species, strain, cell size and form, growth phase and rate, incubation temperature, growth medium composition, pH, osmolarity and aeration, cell water content, lipid content and composition of the cells, density at freezing,

composition of the freezing medium, cooling rate, storage temperature and duration of storage, warming rate, and recovery medium affect the effectiveness of cryopreservation [17].

This work showed that the stored temperature of -80°C was the best temperature for the cryopreservation technique. It is because, at -80 °C, the crystal ice formation is the most stable. There are two kinds of freezing protocols, the controlled (slow) and uncontrolled (fast) ones, which both have been used for fungal cryopreservation. The slow freezing can cause extreme dehydration, and the solution concentration can lead to cell damage. However, fast freezing can lead to insufficient dehydration and formation of abundant ice crystals with lethal consequences [18] and physical damage during storage [19,20]. The temperature where the crystal ice can be formed and cause injury to the cell is somewhere between -5°C and -10 °C. That is why, in this work, the temperature used in the cryopreservation method is -80 °C. Some research have proven that the cryopreservation in the -80°C is suitable for many fungal cultures, including Basidiomycetes [15,21,22,23]. A multitude of protocols has been developed for Basidiomycetes, Ascomycetes, and Zygomycetes [14].

The cryoprotectant used in this work was 10% (v/v) of glycerol and 5% (g/v) of trehalose. It prevents the cell damage caused by the freezing method. Glycerol, as a cryoprotectant, functions as osmoregulation to reduce dehydration during the freezing process. Adding 10% of glycerol and 5% of trehalose could minimize the damage and injury of the cells during the deep freezing process [8]. Glycerol plays a vital role and acts as a cryoprotectant for fungi. It maintains the regeneration activity and prevents cell damage or any lethal consequences — the glycerol functions as osmoregulation during the freezing process. The osmoregulation compounds act to protect cells from significant changes in pressure or osmotic potentials between cells and the environment during freezing. It also reduces the ice crystals formed in the cell cytoplasm [2]. It is the most appropriate preservative used for the long term preservation of fungi. Different fungal cultures exhibit different sensitivities to freezing conditions, existence, and concentration of cryoprotectants [9,14,24,25].

The presence of glycerol as an osmoregulator is more optimal when the trehalose compounds are added [8]. Trehalose is a natural cryoprotective addictive (CPA), which present in plant and yeast cells, and the only disaccharide that has two water molecules in its crystal. The water molecules in the trehalose or the high internal pool of trehalose play a role in protecting the cells during freezing, especially desiccation and heat stress. Besides, the hydroxyl groups of this compound may resemble the dipole of water after dehydration that may allow it to replace the loss of water during cryopreservation. Therefore, it stabilizes the cell condition [8,17].

The other factor that might affect the viability test results is the thawing process. The temperature rate of the thawing process used in this work was 37°C. The rapid thawing is the most preferable because the rapid thawing process in 37°C has shown the least damage to the cell, and provides the best recovery to the cell.

There are several advantages of preserving fungi by freezing technique (provided by freezing and thawing), which are carried out at proper rates and below -80°C storage. The freezing method or cryopreservation has a more straightforward procedure than freeze-drying. In most cases, preservation by freezing will not require protective additive, whereas, in freeze-drying, the additive is needed [17]. The deep-freezing method can be applied to the sporing and non-sporing fungal isolates while maintaining their viability during long term storage. With the freezing method (cryopreservation), genetic stability is maintained thus minimizing physiological and morphological changes [8]. Also, there is no risk of glycerol and trehalose poisoning, simpler process of reviving than the lyophilization because it does not require high lab skills [8,17]. The disadvantages of using a freezing technique for preserving fungi include requirement of adequate tools [14], especially the expensive -80°C deep freezer. It requires a tremendous electrical power for temperature stability. Temperature changes due to electrical power shortage cause the loss of all stored isolates [8].

5. Conclusion

A total of 40 fungal strains from InaCC were tested for their viability and growth rate after three years of storage in the electric deep freezer (-80°C) with 10% (v/v) glycerol and 5% (g/v) trehalose as

cryoprotectant. The results showed that 38/40 (95%) of the observed strains maintain their viability and growth rate. InaCC F226 (Neurospora intermedia) showed the fastest-growing fungal isolate. However, 2/40 (5%) of the observed fungal strains did not grow and result in the viability loss, i.e., InaCC F126 (*Agaricus campestris*) and InaCC F232 (*Xylaria* sp). This study concludes that the cryopreservation technique by freezing at -80ºC is suitable and effectively applied to maintain the viability of filamentous fungal cultures.

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