Accelerated rate storage and viability test of Basidiomycetous fungal strains were cryopreserved at - 80° C

M Ilyas and Y S Soeka

Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences (LIPI) Jl. Raya Bogor Km. 46, Cibinong, West Java, 16911- Indonesia

Email: ilyasmould@yahoo.com

Abstract. Maintaining and preserving microbes such as fungal cultures are essential elements in systematics and biodiversity studies, fundamental part in ex situ conservation and underpinning biotechnology. Majority of Basidiomycetous fungal strains and mushroom cultures in Indonesia are maintained and preserved by serial transfer. This short term active metabolism-based preservation was simple, but it can not be implemented for maintain and storage in the large numbers of fungal cultures due to time consuming and labour intensive. Recently, permanent long term and inactive metabolism-based preservation such as cryopreservation were introduced and implemented for resolved the problems. The objective of this study was to examine the effectiveness of cryopreservation at -80° C with 10% (v/v) glycerol and 5% (g/v) trehalose as cryoprotectant into the 50 strains of the Basidiomycetous fungi from Indonesian Culture Collection (InaCC). The Basidiomycetous fungal cultures were used consists of several taxa such as; Agaricus, Agrocybe, Auricularia, Coprinus, Ganoderma, Lentinula, Phanerochaete, Pleurotus and Trametes. The accelerated storage and viability tests were performed by thawed and revived the stored cultures after 2 weeks and 12 months storage at -80°C. The results showed there are no viability loss among the 50 strains were observed, however there is a distinct acclimatization time and growth rate between cultures were stored at -80°C for 48 hours and more than 3 years. Strains were stored for 48 hours at -80°C begin to grow after 3 days of incubation, whereas cultures were stored at -80°C for more than 3 years begin to grow optimally after 5 days of incubation. Nevertheless, significant differences in acclimatization time and growth rate in both storage periods were not seen in Ganoderma, Phanerochaete, Pleurotus, and Trametes cultures. In general, this long term inactive metabolism-based preservation method is effectively applied for maintain and keep the viability of Basidiomycetous fungal cultures in InaCC.

1. Introduction

Preservation is the act of keeping something in order to prevent it from decaying or to protect it from being damaged or destroyed [1]. Microbial preservation is the act of preserving to keep, maintain, and conserve microbial strains in order to ensure their optimal viability and genetic stability, until they are required for future use [2,3]. In the past, majority of fungal cultures were kept by serial transfer or sub culturing. This methods is simple, inexpensive, and suitable for small collections with cultures in constant use for short periods (less than 1 year). However, sub culturing also has several disadvantages such as time consuming and labour intensive, vulnerable from culture lost through contamination by mites or other microorganisms, mislabelling risk, and the morphology and physiology of a strains may change over time [4,5,6,7].
A wide variety of preservation techniques are available for preserving microbes, however, preservation method in fungi can be distinguish into short term metabolically active and long term metabolically inactive preservation methods [5,7]. Long term metabolically inactive preservation such as freezing or cryopreservation and lyophilization or L-drying are widely acceptable for preserving microbes in long-term period with minimum viability loss and genetic changes[4,7,8].

The choice of preservation method would be implemented depends on the resources i.e. budget, facility, and equipment available, number and concern of microbial would be collected, and also goals of the projects. However, in the standardized microbial culture collection, permanent preservation based on long term inactive metabolism became essential and minimum requirement inorder to guarantee the fungal cultures viability, identity, and characteristic stability. Currently in the standardized culture collection, most of the fungal cultures are stored for long term period using freezing or cryopreservation method. One of the reliable and suitable cryopreservation for fungal cultures especially for non sporulating cultures such as Basidiomycetous fungi are freezing method at -80º C [7, 9,10,11].

Eventhough widely applicable for preserving fungal cultures, in the freezing method at -80º C there is a problem of cells injury during freezing process and thawing and thus, the cultures cannot be revived. Several of cryoprotectants were used for to eliminated the problems, although this is cannot protects the cultures from damage completely. Cryoprotectant were used for freezing consists of two categories; permeating compounds, i.e. glycerol, dimethyl supoxide (DMSO), and non permeating compounds; i.e. sugars, sugar alcohol, dextran, polyvinil pyrolidone [12]. Glycerol and DMSO are the most successful protectants for the cryopreservation of fungal cultures [13].

This study were conducted to determine the accelerated storage and viability test of 50 Basidiomycetous fungal strains from Indonesian Culture Collection (InaCC). They were cryopreserved using freezing method at -80º C in the electric deep freezer with 10% (v/v) glycerol and 5% (g/v) trehalose as cryoprotectant. Preliminary data and information were obtained would be used for examine and evaluate the effectivity of the cryopreservation method.

2. Materials and Methods

Materials were used in this study are 50 Basidiomycetous fungal strains from InaCC, potato dextrose agar (PDA) (Diftco), glycerol stock (Merck), trehalose, ethanol 70%, and distilled water. Consumables were used are 1 ml cryotube, cryotube box, aluminim foil, Petri dish, plastic straw, tooth pick, paper scale, wrapper, scissors, rank, tweezers, stationary, label, plastic, tapes, mask, micro pipette and tip. While the laboratory equipment were used are burner, Beaker glass, measuring glass, scale spoon, hotplate, magnetic stirrer, pH meter, analytical scales, laminar air flow (LAF), water bath, laboratory oven, autoclave, refrigerator 4°C, and electric deep freezer -80°C.

2.1. Fungal medium preparation

Medium were used for fungal culture are potato dextrose agar (PDA). According to [14] PDA plates at 25°C were common and widely used to grown the fungal cultures. Commercially PDA medium (Diftco) were used and then prepared following the manufacturer’s instruction. Measured 39 g of PDA for 1 L distilled water, and then dissolved them with hot plate and magnetic stirrer until completely dissolved and homogenized. Checked and adjusted pH media at 5.6 ± 0.2, and then sterilized using autoclave for 15 minutes at 121°C and 2 atm. Pour PDA medium aseptically into the Petri dish in the LAF after the temperature about 50 - 55°C.

2.2. Cryoprotectant medium preparation

The cryopreservation medium or cryoprotectant were used according to [5] with slightly modification, consists of 10% (v/v) glycerol and 5% (g/v) trehalose. The manufactures of preservation media were first measured 20 ml of glycerol stock and 180 L of distilled water, with measuring glass. Put a them into the Beaker glass and then add 10 g of trehalose. Mix and homogenized the three of materials with hot plate and magnetic stirrer until completely dissolved and homogeneous. Put 1 ml of the solution
into the cryotube and then covering with the screw cap but not too tight. Autoclaved the cryotubes containing 10% (v/v) glycerol and 5% (g/v) trehalose at 121°C, 2 atm for 15 minutes.

2.3. Cryopreservation methods by freezing -80°C
The cryopreservation methods were as described according to [5] with several modification (Figure 1). Inoculated 3 points of fungal strains onto 80-mm Petri dish containing PDA and then incubated at 27°C for 5-7 days incubation (until late logarithmic phase to early stationer phase). Cut fungal cultured aseptically using sterilized plastic straw to form a mycelia and agar disc shaped. Cut the fungi culture in various phase of growth which was in early stationer or late logarithm phase. Put 8-10 discs of mycelial into the cryotube containing 10% (v/v) glycerol and 5% (g/v) trehalose, and then tightly covered the screw cap. Put the cryotubes into cryotube box and acclimatized them in the refrigerator at 4°C for 6 hrs and/or until overnight. After 6 hrs and/or overnight acclimatization at 4°C, moved the cryotube box into the electric deep freezer rack for long term cryopreservation at-80°C.

2.4. Accelerated rate storage and viability test
The viability test and purity test were done according [15] with several modification (Figure 2). Picked up the cryotubes containing fungal cultures from the deep freezer -80°C and then thawed immediately in the water bath at 37°C for 3 min. After thawing, the bead or disc shaped cultures in the cryotube were 3 points inoculated onto PDA media. Cultures then incubated at 27°C for 5-10 days and during incubation period, examines the fungal growth and the growth rate by measuring the colony diameter.

![Cryopreservation procedure](image-url)
3. Results and Discussion

The cryopreservation method for preserving fungal cultures were conducted by lowering the temperature of biological material in order to reduces the rate of metabolisms until below the frozen point. Furthermore when all internal water in cellular protoplasm is frozen or reach glass temperature there will no further biochemical reaction occur and the metabolism will be suspended [5]. The result of accelerated rate storage and viability test result of 50 InaCC Basidiomycetous fungal strains after 48 hours (ARST) and 3 years storage in electric deep freezer -80° C were shown on Table 1.

Table 1.Viability and growth rate average of 50 InaCC Basidiomycetous fungal strains after 48 hours (ARST) and 3 years storage in electric deep freezer -80° C

| No  | InaCC no. | Fungal taxa                        | Growth rate/ colony diameter (cm) |
|-----|-----------|-----------------------------------|-----------------------------------|
|     |           |                                   | ARST (48 h)                        | >3 years storing |
|     |           |                                   | 72 h | 120 h | 72 h | 120 h |
| 1   | InaCC-F9  | Auricularia polytricha (Mont.) Sacc. | 0.4  | 2     | -   | 0.5  |
| 2   | InaCC-F10 | Pleurotus ostreatus (Jacq. ex Fr.) P.Kumm. | 1    | >3    | 0.8 | 2.3  |
| 3   | InaCC-F11 | Ganoderma lucidum (Curtis) P. Karst | 0.6  | 2.8   | 0.5 | 2.6  |
| 4   | InaCC-F12 | Pleurotus ostreatus (Jacq. ex Fr.) P.Kumm. | 0.8  | >3    | 0.8 | >3   |
| 5   | InaCC-F19 | Pleurotus eryngii (DC.) Quél.       | 0.4  | 1.2   | -   | 0.5  |
| 6   | InaCC-F95 | Lentinula edodes (Berk.) Pegler, Kavaka | 0.3  | 1     | -   | 0.5  |
| 7   | InaCC-F97 | Pleurotus ostreatus (Jacq. ex Fr.) P.Kumm. | 1    | >3    | 0.6 | 2.8  |
| 8   | InaCC-F100| Pleurotus cystidiosus O.K. Mill.    | 0.6  | 2.5   | 0.4 | 2    |
| 9   | InaCC-F104| Pleurotus ostreatus (Jacq. ex Fr.) P.Kumm. | 0.8  | 2.8   | 0.8 | >3   |
Table 1 showed that there are no viability loss among the 50 strains were observed, however there is a distinct acclimatization time and growth rate between cultures were stored at -80°C for 48 hours (ARST) and more than 3 years. Strains were stored for 48 hours at -80°C begin to grow after 3 days of incubation, whereas cultures were stored at -80°C for more than 3 years begin to grow optimally after 5 days of incubation. Nevertheless, significant differences in acclimatization time and growth rate in both storage periods were not seen in *Ganoderma, Phanerochaete, Pleurotus,* and *Trametes* cultures. The growth rate were examined by measuring colony diameter could be conducted generally after 3 days incubation period (Figure 3).
In general, freezing method at T -80°C with 10% (v/v) glycerol and 5% (g/v) trehalose as cryoprotectant are suitable for maintaining the viability of 50 Basidiomycetous fungal strains which were observed. Cryopreservation by freezing at -80°C is suitable for many fungal cultures, including Basidiomycetes [6,16,17,18]. Cryopreservation also the most reliable long-term preservation method used for fungi and a multitude of protocols have been developed for fungal strains from taxa Basidiomycetes, Ascomycetes and Zygomycetes [19].

There are several factors that will affected the effectiveness of cryopreservation in microbial cultures such as; species, strain, cell size and form, microbial growth phase, temperature for incubation, 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 [20]. Fungal cultures may also lost their viability during freezing because of repeating process during freezing and thawing which will significantly reduce the viability of the cultures [7]. According to [19] reported that the viability rate of cryopreserved fungi mainly depends on the cryoprotectants were used, the freezing rate, and the thawing rate.

The cryopreservation seems to be the best preservation technique were available for filamentous fungi [16]. The cryopreservation process includes freezing and thawing and the protocol of these procedures plays an important role. There are two kinds for freezing protocol, i.e. slow or controlled freezing and fast or uncontrolled freezing protocol. The slow freezing rates can cause an excessive dehydration and concentration of the solution leading to cell damage. However, instant or too fast freezing leads to insufficient dehydration and formation of abundant ice crystals with lethal consequences [21]. According [22,23,24], freezing method can cause the formation of extracellular crystal ice which has irregular shape that can lead to physical damage during storage. The temperature
where mostly the crystal ice can be formed and can caused injury to the cell is somewhere between -5°C and -10°C.

Nevertheless, different fungal cultures showed different sensitivity to the freezing conditions and to the presence and concentration of cryoprotectants [21]. According to [13] another factor for successfulness of cryopreservation in fungal cultures depends on the cryoprotectant were used. The cryoprotectant were used in this study consists of 10% (v/v) of glycerol and 5% (g/v) of trehalose. Glycerol plays an important role and acts as a cryoprotectant for fungi, it maintains regeneration activity and prevent the cells from damage could minimalize the damage and injury of the cells during the deep freezing process. The glycerol compound has a function as an 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 process. Glycerol also serves to reduce the formation of ice crystals in the cell cytoplasm [14]. Glycerol is most appropriate preservative for the long term preservation of fungal cultures [5,19,24,25].

The presence of glycerol as cryoprotectant will be more optimal if the trehalose compounds were added. Trehalose is a natural cryoprotective additive or CPA, which present in plant and yeast cells, and the only disaccharide that has two water molecules in its crystal [20]. The water molecules in the trehalose play a role in protecting the cells during freezing, especially desiccation and against heat stress. In addition, the hydroxyl groups of this compounds may resembles the dipole of water after dehydration. This may allow it to replace the loss of water during cryopreservation, therefore, it could stabilize the cell condition. Trehalose as cryoprotectant also can stabilized the cytoplasmic membrane and liposome [26].

Another factor that might be affected the viability test results is the thawing process. According to [5] the rapid thawing is the most favorable or preferable for reviving the cryopreserved fungal strains. Rapid thawing process in the temperature of 37°C showed the least damage or injury to the cell, and it gives the best recovery to the cell.

4. Conclusion
Preservation and maintenance of fungi for long term period is a basic need for research and industrial purpose. Freezing or cryopreservation are method for the microbial preservation were conducted by lowering the temperature below the freezing point. In this study 50 representative Basidiomycetous fungal strains from InaCC, were being tested for their viability and growth rate after 48 hours and more than three years storing in the electric deep freezer at -80°C with 10% (v/v) glycerol and 5% (g/v) trehalose as cryoprotectant. The results showed there are no viability loss among the 50 strains were observed, however there is a distinct acclimatization time and growth rate between cultures were stored at -80°C for 48 hours and more than 3 years. Strains were stored for 48 hours at -80°C begin to grow after 3 days of incubation, whereas cultures were stored at -80°C for more than 3 years begin to grow optimally after 5 days of incubation. Nevertheless, significant differences in acclimatization time and growth rate in both storage periods were not seen in Ganoderma, Phanerochaete, Pleurotus, and Trametes cultures. In general, this long term inactive metabolism-based preservation method is effectively applied for maintain and keep the viability of Basidiomycetous fungal cultures in InaCC.

5. References
[1] Cambridge 2011 Cambridge Essential English Dictionary 2nd ed. (Cambridge, United Kingdom: Cambridge University Press)
[2] Onions A H S 1971 Preservation of fungi. In: Methods in Microbiology C. Booth(ed.), Vol. 4, (London: Academic Press) pp 113-151
[3] Smith D and Ryan M 2012 Implementing best practices and validation of cryopreservation techniques for microorganisms The Scientific World Journal 1-9
[4] Smith D 1983 Cryoprotectants and the cryopreservation of fungi Transactions of the British Mycological Society 80(2) 360-363
[5] Smith D and Onions A H S 1994 The Preservation and Maintenance of Living Fungi. 2nd ed. IMI Technical Handbooks (Wallington UK: CAB International)
[6] Kitamoto Y, Suzuki A, Shimada S, and Yamanaka K 2002 A new preservation method of fungus stock cultures by deep freezing Mycoscience 43(2) 143-149
[7] Nakasone K K, Peterson S W, and Jong S-C 2004 Preservation and distribution of fungal cultures In: Biodiversity of Fungi: Inventory and Monitoring Methods. Mueller, G.M., G.F. Bills, and M.S. Fosters (eds.) (Burlington, MA USA Elsevier Academic Press) pp 37-47
[8] Humber R A 1997 Fungi: Preservation of cultures. In: Manual Techniques in Insect Pathology. Lacey L.A. (ed.) (San Diego: Academic Press) pp 269-279
[9] Ito T and Yokoyama T 1983 Preservation of basidiomycete cultures by freezing. IFO Research Communication 11 60-70
[10] Ito T and Nakagiri A 1996 Viability of frozen cultures of Basidiomycetes after fifteen year storage Microbiological Culture Collection 12 67-78
[11] Palacio A, Gutiérrez Y, Rojas D, Atehortúa L, and Zapata P 2014 Viability of Basidiomycete fungal strains under different conservation methods: cryopreservation vs. freeze-drying processes Actualidades Biologicas 36 (100) 13-21
[12] Corbery Y and Tacon F L 1997 Storage of ectomycorrhizal fungi by freezing Annual Science Forum 54 211-217
[13] Jong S C and Davis E E 1978 Conservation of reference strains of Fusarium in pure culture Mycopathologia 66 153-159
[14] Paul J S, Tiwari K L and Jadhav S K 2015 Long term preservation of commercial important. fungi in glycerol at 4°C International Journal of Biological Chemistry 9 (2) 79-85
[15] Espinell-Ingroff A, Montero D and Martin-Mazuelos E 2004 Long-term preservation of fungal isolates in commercially prepared cryogenic microbank vials Journal of Clinical Microbiology 42(3) 1257-1259
[16] Smith D 1998 The use of cryopreservation in the ex situ conservation of fungi Cryo Letters. 19 79-90
[17] Stummer B E, Zanker T, and Scott E S 1999 Cryopreservation of air dried conidia of Uncinula necator Australia Plant Pathology 28 82-84
[18] Singh S K, Upadhyay R C, Kamal S, and Tiwari M 2004 Mushroom cryopreservation and its effect on survival, yield and genetic stability Cryo Letters 25 23-32
[19] Lalaymia I, Cranenbrouck S, and Declerck S 2014 Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi Mycorrhiza 24 323-337
[20] Hubalek Z, 2003 Protectants used in the cryopreservation of microorganisms Cryobiology 46 205-229
[21] Gupta V K, Tuohy G M, Ayyachamy M, Turner M K, and O’donovan A 2011 Laboratory Protocols in Fungal Biology: Current Methods in Fungal Biology (New York: Springer Science & Business Media)
[22] Hwang S W 1969 Effects of ultra-low temperatures on the viability of selected fungus strains Mycologia 52(3) 527-529
[23] Fennell D I 1960 Conservation of fungous cultures. Botanical Review 26(1) 79-141
[24] Hwang SW 1966 Long-term preservation of fungus cultures with liquid nitrogen refrigeration Appl. Environ. Microbiol. 14 784-788
[25] Hwang S W 1968 Investigation of ultra-low temperature for fungal cultures: An evaluation of liquid-nitrogen storage for preservation of selected fungal cultures Mycologia 60 613-621
[26] Roser B 1991 Trehalose, a new approach to premium dried foods. Trends Food Science Technology 2 166-169

Acknowledgments
This study was supported by DIPA thematic project of Research Center for Biology, Indonesian Institute of Sciences (LIPI). Authors would like to express special gratitude and thanks to Mrs. Yeni Yuliani, Sahal Muadz, and M.N. Ruwandani for sincere technical work support in this study.