Isolation and preservation of microfungi from Indonesia islands

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Abstract. The research was aimed at utilizing more adequate methods of long-term preservation, cryopreservation (freezing at -80 °C). Samples obtained from three islands were used as sources of samples, i.e., Enggano, Riau, and Sumba. Direct isolation and single spore isolation were conducted to isolate the microfungi. All the cultures were grown on Potato Dextrose Agar (PDA). The growth rate of selected microfungi was calculated every day for a week. The growth rate for selected ten microfungi of each island, approximately 0.1-0.7 cm/day. All the cultures are maintained in InaCC (Indonesian Culture Collections), Cibinong, LIPI.

1. Introduction

The microfungi are the most diverse group of all the fungi, but the least understood or documented. Only about 5-10% of all fungal species have been described, much less characterized and put to use or controlled. Investigations to explore the diversity of microfungi have shown that they are much more diverse than previously thought. Very small samples of tropical rainforest leaf litter yielded up to 145 different species of microfungi [1].

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 [2]. Based on the period and objective, the microbial conservation and storage were distinguished into (1) short-term, for a short period of time, such as from isolation until correct identification is done; (2) intermediate-term, such as the duration of a research project, and (3) long-term, for collection, conservation, or research references [3]. In principle, there are two kinds of freezing protocols: a slow (controlled) one and a fast (uncontrolled) one, which both have been used for cryopreservation of fungi. Nevertheless, different fungal cultures exhibit different sensitivities to freezing conditions and the presence and concentration of cryoprotectants. Some reports indicate that cryopreservation at -80°C is suitable for many fungal cultures, including basidiomycetes.

Cryopreservation is a technique preserved by lowering the temperature of biological material reduces the rate of metabolism until all internal water is frozen and no further biochemical reactions occur [4]. Cryopreservation storage is now in place for many important collections of algae, cell cultures, and organized plant tissues, and techniques are now available for many more [5]. 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 [6]. The objective of the research is to preserve the microfungi from Indonesia.
2. Materials and Methods

2.1. Isolation method and sources of samples
The microfungi were isolated by single spore isolation methods [7]. The source locations of microfungi were from the outer islands of Indonesia (Sumba, Enggano, and Riau) Islands.

2.2. Preparation of Potato Dextrose Agar Medium
Potato Dextrose Agar medium was prepared to follow the instruction from the company. After that, the PDA medium was poured into a Petri dish with an aseptic technique in the Laminar Air Flow (LAF). After solidifying, the Petri dish was arranged in a ziplock and stored in a clean room for further use.

2.3. Preparation of Cryoprotectant
The cryoprotectant medium consists of 10% glycerol, 5% trehalose, and pure water 180 mL. The procedures for making the cryoprotectant solution follow Susilawati [8].

2.4. Labeling
The label was created with size 2x4 cm; consist of isolate code and date of the isolate.

2.5. Thawing and Reviving
To maintain the availability, purity, and identity, thawing and reviving is needed. The frozen culture was taken from a refrigerator at temperature -80 °C. The frozen culture was thawed quickly in a 37°C water bath and transfer immediately to PDA media with Duplo method. The purpose was to observe the cultural character and to increase the amount of culture. The transfer process occurred on laminar airflow with an aseptic technique to prevent contaminant. The thawed agar plugs were taken from cryotube by the sterile skewer and placed into Petri dish then wrapped the Petri dish and incubated at an appropriate temperature.

2.6. Morphology and molecular identity (for selected cultures)
The cultures should be checked for every two days. The cultural characters, which consist of growth rate, surface, margin, color, form, elevation, water drops, and the aerial hyphae for each isolate were observed. The molecular is needed to confirm the identity after preservation in the next work.

2.7. Preservation
The preservation process able to do after the culture was incubated at least one week. The preservation equipment was prepared in the Laminar Air Flow (LAF). The disk of the isolate was made by sterile straw and inserted to cryotube, which has been filled with the cryoprotectant medium.

3. Result and Discussion
The preservation of microfungi from outer island of Indonesia including Enggano island, Sumba island and Riau archipelago with cryopreservation technique using cryoprotectant. The result of the preservation of microfungi by using growth rate parameter, which counted per two days. The list of the fungi that have successfully cultivated in the artificial media was listed below: The Sumba fungi were: SM8, SM7, SM41, SM35, SM33, SM36, SM26, SM28, and SM 12; The Riau fungi were: PK21, PK30, PK09-01, PK17-01, PK09-02, PK10, PK12, PK04-01, PK07, and The Enggano fungi were: KK3B, KP2A, 3K504, KK3K3, KP2B2A, KP1A, MER2, CAS2, KKB, and IK2.

The growth rate of microfungi of Enggano island showed the highest growth rate was KK3B and 3K504 with 1.5 cm/2 day while the lowest growth rate was KKB with 0,25 cm/2 day (Figure 1). The highest growth rate of microfungi from Sumba island were SM14 and SM36 with 1 cm/2 day, while the lowest SM9 with 0,1 cm/2 day (Figure 2). The highest growth rates of microfungi from Riau Archipelago were PK09-02 with 0,75 cm/2 day while the lowest PK17-01 with 0,33 cm/2 day (Figure was not shown).
Based on the entire growth of microfungi from those islands the highest growth rate is KK3B, 3K504 with 1.5 cm/2 day from Enggano island, while the slowest growth has lowest of growth rate is SM12 with 0.1 cm/2 day from Sumba island. Based on the resulting growth of fungi on media showed that the ability of fungus after the preservation was good. The fungi grow on artificial medium forming colonies of the different shape, size, and color. Size of colonies enhances day by day attaining the full growth at a definite period. However, fungal growth can be estimated by measuring the size of colonies per hour or per day. Type of culture media and their chemical compositions significantly affected the mycelia growth rate and conidial production. Most fungi thrive on PDA, but this can be too rich in nutrients, thus encouraging the mycelial growth with the ultimate loss of sporulation. Conventionally, starch-based media, such as PDA or MEA are good substrates for the species fungus, which grow rapidly and produce abundant aerial mycelia. Glucose, usually an excellent carbon source for growth, interfered with the biosynthesis of many secondary metabolites [9].
Figure 2. Culture from Enggano island (KK3B). The cultural characteristics (margin filiform; surface wooly; form circular and elevation flat, aerial hyphae with no water drop; the surface white-pinkish); The growing rate 1.5 cm/2 day

Figure 3. Culture from Enggano island (3K504). The cultural characteristics (margin filiform; surface cottony; form circular and elevation flat, aerial hyphae with no water drop; the lower and upper: white-greyish); The growing rate 1.5 cm/2 day

Figure 4. Culture from Sumba islands (SM12). The cultural characteristics (margin undulate; surface velvety; form irregular; elevation convex; aerial hyphae with no water drop; the surface olivaceous green); The growth rate 0.1 cm/2 day

Fungi collected, isolated dan preserved in this paper were mostly ascomycota and phytopathogenic fungi that different from the fungi collected before by Kramadibrata [11] and Susan [10]. Based on the result of mushroom exploration on Enggano Island by Susan [10], obtained 31 species of macrofungi belonging two phyla, three classes, nine order and fifteen families, most of the collection by Susan, were phylum Basidiomycota and Ascomycota, and some of the fungi were new records and new potency as color degradation agents. Kramadibarata recorded twelve arbuscular fungi identified from a variety of good locations; coastal forest, land forest and garden. Unfortunately, there was no information about the isolation and cultural preservation of Basidiomycota, Ascomycota and arbuscular fungi collected from Enggano island, as collected by Kramadibrata and Susan [11, 10]. In 2018, Lestari [12] mention the indigenous fungi from Riau, namely Penicillium sp. PN6 will improve percentage reduction in Total Proteleum Hydrocarbon by using Neptunia oleracea combination. Lestari [13] recorded fourteen fungi belonging to the Basidiomycota isolated from Riau Province by using Phylogenetic analyses. Again, there was no information about cultural preservation of the fungi from Riau island as recorded by Lestari and Lestari [12,13]. Fungi from Sumba island recorded many as AF (Arbuscula Fungi) [14]. All these fungi was also no information about culture preservation and or isolation.

Fungi are very important for the environment and part of human lives. The importance of fungal preservation to keep the viability, purity, and identity of the fungi. The Fungi will have benefit for agriculture-forestry, environment, medicine-antibiotics and pharmaceutical [15,16].
4. Conclusion
The use of preservation of fungal species is important for long time use in industries and research work, one of the methods that mostly used for long-term storage is cryopreservation. The successful preservation of fungal strains is the maintenance of their genetic and physiological features, such as growth, morphology, and metabolite production. Based on the reviving the growth of fungal was measured by growth rate. Most of the isolates are growing well especially isolate KK3B, 3K504 and BS1 showed the fastest growth rate. All thirty cultures will be store in InaCC, Indonesia. For further research can be applied using liquid nitrogen or lyophilization. The preservation of the fungi for better future science is very important, especially for Indonesia.

5. References
[1] Bills G F and Polishook J D 1994 Abundance and diversity of microfungi in the leaf litter of lowland rain forest in Costa Rica Mycologia 86 187-198
[2] Paul J S, Tiwari K L and JadHAV S K 2015 Long Term Preservation of Commercial Important Fungi in Glycerol at 4 °C J. Biol. Chem 9(2) 79-85
[3] Machmud M 2011 Teknik Penyimpanan dan Pemeliharaan Mikroba Bull. AgroBio 4(1) 24-32
[4] Homolka L. 2013 Method of Cryopreservation in Fungi. In: Laboratory Protocol in Fungal Biology: Current Method in Fungal Biology (New York; Springer New York) pp 9-16
[5] Ryan J M and David S 2011 Cryopreservation and Freeze-Drying of Fungi Employing Centrifugal and Self Freeze-Drying 368.(Totowa: Humana Press Inc NJ)
[6] Nakasone K K, Peterson A W and Jong S 2004 Preservation and Distribution of Fungal Cultures.In: Mueller, G. M.; Bills, G. F.; Foster, M. S. Biodiversity of fungi, inventory, and monitoring methods. San Diego: Elsevier, p. 37-47
[7] Wulandari N F, To anun C, Hyde K D, Duong L M, de Gruyter J, Meffert J P, Groenewald J Z, and Crous P W 2009 Phyllosticta citriasiana sp. nov., the cause of Citrus tan spot of Citrus maxima in Asia. Fungal Diversity 34 23-39
[8] Susilawati L and Purnomo E S 2016 Viabilitas Sel Bakteri Dengan Cryoprotectant Agents Berbeda (Sebagai acuan dalam preservasi culture collection di Laboratorium Mikrobiologi) Biogenesis 4(1) 34-40
[9] Pradeep F S, Begam M S, Palaniswamy M, Pradeep B V 2013 Influence of Culture Media on Growth and Pigment Production by Fusariummoniliforme KUMBF1201 isolated from Paddy Field Soil World Appl. Sci. J. 22(1) 70-77
[10] Susan D and Retnowati A 2017 Catatan Beberapa Jamur Makro dari Pulang Enggano: Diversitas dan Potensi dari [Notes on Some Macro Fungi from Enggano Island: Diversity and Its Potency] Berita Biologi 16(3) 243-256
[11] Kramadibrata K 2016 Keaneakaragaman Jamur Arbuskula di Pulau Enggano [Diversity of Arbucular Fungi in Enggano Island] Berita Biologi 15(3) 207-319
[12] Lestari W, Martina A, Roza R M, and Wardani I 2018 Potensi Jamur Indigensus Riau (Penicillium sp. PN6) dan Neptuna oleracea untuk Bioremediasi Oil Sludge [Riau Indigenous Fungi (Penicillium sp. PN6) and Neptuna oleracea Potention in Bioremediation of Oil Sludge] Al-Kauniyah: Journal of Biology 11(1) 72-81
[13] Lestari A S L, Zulfiana D, Zulfriti A, Krishanti N P R A and Titik Kartika 2018 Phylogenetic Analysis of Polyoporous Fungi Collected from Batam Botanical Garden, Riau Province, Indonesia Biosainitika 10(3) 510-518
[14] Husna, Budi S W B R, Mansur I, Kusmana C and Kramadibrata K 2014 Fungi Mikoriza Arbuskula pada Rizosfer Pericopsis mooniana (Thw.)Thw. di Sulawesi Tenggara [Arbuscular Mycorrhizal Fungi from Rhizosphore of Pericopsis mooniana (Thw.)Thw.] in South-East Sulawesi] Berita Biologi 13(3) 263-273
[15] Beekman A M and Barrow R A 2014 Fungal Metabolites as Pharmaceuticals Aust. J. Chem 67 827-843.
[16] Svan K S, Goranson U, El-seedi H, Bohlin L, Larson J, Olsen B, and Chryssanthou E 2012 Antimicrobial activity of filamentous fungi isolated from highly antibiotic-contaminated river sediment Infec. Ecol. Epidemiol 2 11591
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