Chapter 3
Establishment and Management of Culture Collections of Microorganisms (mBRC): An Overview

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Abstract Microbes are the most diverse of all living organisms and play crucial roles in nutrient cycling that support all life forms on Earth. The conservation and preservation of microbes are important for the life sciences, especially the development of sustainable agriculture. This chapter introduces the main requirements for establishing and operating a general public microbial bioresource center, including funding, holdings, facilities, and staff, as well as maintaining, depositing, and distributing microbial cultures. Updated multi-technical aspects of the preservation of bacteria, actinomycetes, fungi, mushroom, microalgae, and microbial communities, in reference to some potential applications in agriculture, are discussed, such as using fungi and mycorrhizae for short-, medium-, and long-term preservation. Additionally, current international collaborations are discussed, including access and benefit sharing of microbial genetic resources under the Convention on Biodiversity and Nagoya Protocol in Asian countries under the Asian Consortium for the Conservation and Sustainable Use of Microbial Resources. A case study on the Nagoya Protocol on access and benefit sharing in a bilateral collaboration between the National Institute of Technology and Evaluation, Japan, and the Institute of Microbiology and Biotechnology, Vietnam National University, Vietnam, is also evaluated.

3.1 Introduction

Microbes are microscopic organisms, including bacteria, actinomycetes, fungi, viruses, and algae. Microbes form most of the biomass on the Earth and play important roles in biogeochemical processes that are important for the survival of all life forms (Sharma et al. 2017a). The discovery of the first microorganisms was made possible by the work of Antonie van Leeuwenhoek in 1677. However,

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S. K. Sharma, A. Varma (eds.), *Microbial Resource Conservation*, Soil Biology 54,
https://doi.org/10.1007/978-3-319-96971-8_3

microbial activities and functions were made evident through the study of lactic acid fermentation by Pasteur (1822–1895) and the study of anthrax by Koch (1876). Rhizobia were isolated and identified by Beijerinck (1888), and microorganisms were recognized as important to humans.

Culture collections were first established in European countries. Frantisek Král (1846–1911) established the first culture collection of bacteria in 1890 at the German University, Prague, and it is the oldest culture collection in the world. In addition, the Culture collection of the Pasteur Institute (1891, France), the Mycothèque de l'Université catholique de Louvain (1982, Belgium), the Centraalbureau voor Schimmelcultures (CBS; 1904, Netherlands), and the Centre for Agriculture and Biosciences International (CABI; 1910, UK) were established. Traditionally, culture collections focus on a single particular group of microbes, such as bacteria, fungi, or entomopathogens.

The Organization for Economic Cooperation and Development (OECD) established the concept of a Biological Resource Center (BRC). BRCs are both service providers and repositories of the living cells, genomes of organisms, and information relating to heredity and the functions of biological systems. BRCs contain collections of cultivable organisms (microorganisms, plants, animals, and human cells), replicable parts of these organisms (genomes, plasmids, viruses, and cDNAs), viable but not yet cultivable organisms' cells and tissues, as well as databases containing molecular, physiological, and structural information relevant to these collections and related bioinformatics (OECD 2007).

As the value of microbial BRCs (mBRCs) has become apparent (Stern 2004), the number of service collections, now greater than 600, has increased. The register of culture collections at the World Data Centre for Microorganisms (WDCM) has data on almost 746 culture collections worldwide that hold over 2.6 million strains of microorganisms, mainly bacteria and fungi. Almost 130 of these are collections affiliated with the World Federation for Culture Collections (WFCC), and as such, they operate under WFCC guidelines (Smith et al. 2014).

3.2 Establishment

3.2.1 Necessity and Impact of mBRCs in the Life Sciences

3.2.1.1 Necessity

Microbes were likely the first forms of life to develop on Earth, approximately 3–4 billion years ago, and were involved in the development of the living world (Schopf 2006). Microbes can live in extreme ecosystems, such as hot spring and very cold areas, as well as in acidic and alkali environments. Microbes likely played a role in the Earth's atmosphere changing from supporting anaerobic to aerobic life and, directly or indirectly, in the evolution of the living world. Microbes are involved in many element (N, C, P, S, and more) cycles. There have been recent revolutions in

various fields of science and technology, such as molecular biology, nanobiotechnology, and bioinformatics, and these, along with new materials, provide humans more power to address many problems, such as climate change, food security and safety, and virulent diseases, such as influenza A and influenza Zika. To overcome these problems, maintaining and utilizing biodiversity are crucial. Microbes are much more diverse than plants and animals. The combined number of plant and animal species is estimated as 2–50 million (https://www.factmonster.com/science/animals/estimated-number-animal-and-plant-species-earth), but the figure for microbes is ~1 trillion (http://www.newsweek.com/new-estimate-there-are-1-trillion-species-microbes-earth-454714). At present, most animal and plant species have been identified and characterized, but, for microbes, only an estimated 0.1–10% has been identified and characterized (Kennedy 1999). However, microbes make great contributions to the life sciences. In medical and pharmaceutical fields, the most important application of microbes is in antibiotics. Flemming isolated the first antibiotic from *Penicillium* in 1928 (Tan and Tatsumura 2015). At present, ~16,500 antibiotics have been isolated from microbes, mainly from actinomycetes (~8700) and fungi (~4900) (Bérdy 2005). In addition, more than 4000 bioactive compounds, including amino acids, have various useful applied activities, such as antiviral and anticancer, as well as fungicidal and bactericidal. The global antibiotic market alone reached more than an estimated 1000 billion USD in 2014 (http://www.abpi.org.uk/industry-info/knowledge-hub/global-industry/Pages/industry-market-.aspx#fig 2). Presently, humans are facing problem of antibiotic resistance in microbes. Thus, identifying new antibiotics and using new microbial biotechnologies are important for protecting humans.

In Industrial Applications

Microbes, directly and indirectly, are being used for various applications in industry, such as in brewing and food processing, and in cosmetic and dairy products, such as yogurt and fermented foods. The global market value of microbe-related industries is more than 1000 billion USD. The global beer market alone was valued at approximately 530 billion USD in 2016 and is expected to reach approximately 750 billion USD by 2022 (https://www.zionmarketresearch.com/report/beer-market).

Agricultural and Environmental Applications

According to the US Department of Agriculture (USDA), global rice and grain productions were ~480.3 and 680 million tons in 2016, with a value of more than 3000 billion USD (https://apps.fas.usda.gov/psdonline/circulars/grain-rice.pdf). In addition, some other agricultural products can be used for industry, such as coffee, cacao, rubber, and tea, to satisfy human demands. The biodiversity of microbes has crucial roles in agricultural production, and they are important factors for the bioconversion involved in all important element cycles, such as those of C, N, P, and S. Through their biological activities, many mineral elements and nutrients are provided for agricultural cultivation. Lignocellulose from plants is the most abundant organic carbon source in the biosphere. For biomass production, the plant needs many elements, including C, N, P, K, and S, from the environment. In sunlight, the plant can biosynthesize lignocellulose, but significant amount of soluble organic C

and N comes from the soil as microbial biomass and the degradation products of microbial organic materials. Many groups of microbes (such as *Azotobacter* and *Azospirillum*) can fix atmospheric nitrogen in a soluble form that is easily assimilated by plants. Phosphorus, potash, and sulfur exist mostly in insoluble forms in the soil, but some microbes can solubilize and mineralize these elements for plants using various manners, such as organic acid production (acetic, gluconic acid, malic acid, alpha-ketoglutaric acid, and lactic acids) or enzymes (phosphatase and phytase). *Mycorrhizae* improve plant growth by the development of symbiotic relationship with the roots of 95% of plant families and thereby aid in nutrient exchange, increase disease and drought resistance, and ultimately reduce the need for chemical fertilizers. Additionally, they can produce some useful hormones that stimulate plant growth and maintain a good soil structure as carriers for many nutrients and microflora that maintain micro-ecosystems. Presently, humans are facing problem of climate change, and resulting in reduction in agricultural areas in the many parts of the world due to rise and of sea level. The top countries at risk from the negative impacts of climate change belong to Africa and Southeast Asia, including Zimbabwe, Madagascar, Mozambique, Cambodia, Vietnam, and the Philippines as well as other countries, such as Haiti and Bangladesh. The Vietnamese Ministry of Resources and Environment estimates that when the sea level increases by 1 m, the agricultural area in the Cuu Long River Delta of Vietnam will be reduced by 30–40%. The annual rice production will be decreased by 7.6 million tons, and the gross domestic product will be reduced by 10%. In addition, the world's population is dealing with food security and safety issues. With revolutions in the sciences, especially in molecular biology and technology, many new varieties of plants, including genetically modified ones that can grow in extreme conditions, such as saline and sandy soils, have been created, and technology has been applied to agriculture to increase food production. In addition, many kinds of microbial-based fertilizers have been developed to enhance productivity by improving the quality of grain and nutrients available in cultivated soils. The biodiversity and numbers of microbes in soil are strong indicators of its quality and bioproductivity.

Food-borne diseases are risks to humans. Various diseases are related to the accumulation of chemical pesticides and fertilizer residues on food. The use of chemical fertilizers and insecticides in agriculture is decreasing. Various kinds of microbes, including bacteria, yeast, microalgae, and actinomycetes, are now used for microbial fertilizer production. By using microbial fertilizers, the amount of chemical fertilizer used can be decreased by 52% (Gray et al. 2013). The yield of plants and crops is treated for biodisasters caused by mainly insects and pathogenic microbes. Overuse of chemical pesticides is a main reason for human liver and kidney diseases. Many microbes such as *Beauveria*, *Metarhizium*, *Chaetomium*, *Paecilomyces*, *Lecanicillium lecanii*, *Bacillus thuringiensis*, *Bacillus subtilis*, and *Streptomyces* (Usta 2013) have been used successfully as bioinsecticides, fungicides, and bactericides. The trend in green and sustainable agricultural development has gained the public's attention; thus, qualified biofertilizers and pesticides are being developed. The global bioinsecticide market is estimated to reach a value of 3.18 billion USD by

2021. The market is driven by factors such as increasing levels of pest attacks due to climate change and the implementation of biological technologies for pest control (http://www.marketsandmarkets.com/PressReleases/bioinsecticides.asp). The global biofertilizers' market size was estimated at 535.8 million USD in 2014. The agricultural countries like India, China, Vietnam, and the United States introduced national initiative aims at increasing biofertilizer production, distribution, and utilization, which spurred the biofertilizers' market growth. In the United States, the biofertilizer market value was ~140 million USD in 2017, but the figure is expected to reach ~280 million USD in 2022 (http://www.grandviewresearch.com/industry-analysis/biofertilizers-industry).

The world population will reach 7.5 billion in 2017 (http://www.worldometers.info/world-population/), compared with 6.1 billion in 2000. The increase in the human population will result in increasing demands for food, healthcare, energy, and transportation. Many countries are facing urbanization and industrialization, resulting in heavy environmental pollution. Pollution, especially that caused by organic sources, is one reason for the outbreak of various kinds of viral diseases, such as *Vibrio chlorella*, *Escherichia coli* H157, influenza A, and malaria. To address this problem, progress in environmental technology and in the sciences has been made. Microbial products are applied in a variety of these technologies to help treat environmental pollution.

3.2.1.2 Impacts of mBRCs

As with plants and animals, or any organism, microbes are a national resource. The diversity level of microbes has potential for the development of the life sciences. Because mBRCs are part of the important infrastructure for the life sciences and biotechnology, they play several main functions.

Ex Situ Conservatories of Microbes and Custodians of National Microbial Biodiversity

There are three levels to biodiversity, gene, species, and ecosystem. The megadiverse countries are a group of 17 countries, Australia, Brazil, Colombia, the Democratic Republic of the Congo, China, Ecuador, India, Indonesia, Madagascar, Malaysia, Mexico, Papua New Guinea, Peru, the Philippines, South Africa, the United States, and Venezuela, that harbor the majority of the Earth's species and have high numbers of endemic species. Some of these countries are located in, or partially in, tropical or subtropical regions. However, biological diversity has been reduced significantly by certain human activities, mainly industrialization and urbanization. Microorganisms are not only of value for the production of useful substances, they also play unique roles in element cycles with plants and animals. Microorganisms are also the sources of significant gene pools for research and applications in various fields. In addition, a healthy environment requires green and sustainable economic development. Microbial biodiversity is crucial for this goal and should not be lost. Thus, microorganisms can be regarded as part of the

cultural heritage and property, and they must be transferred to the next generation in a normal and healthy condition (http://www.iupac.org/symposia/proceedings/phuket97/komagata.html).

Providing Qualified Microbes as Starting Genetic Resources for Scientific, Agricultural, Environmental, Medical, and Pharmaceutical Research and Applications

Maintained microbes are not only preserved but used. Microbes, directly and indirectly, are used in almost all fields related to the life sciences and biotechnology. Scientific investigations and applications must be reproducible. Experiments performed in one laboratory by one set of investigators and facilities replicable in another laboratory. Reliable standardized information on microbes can be very important for particular experiments or applications to be conducted. Microbes are very diverse, but their roles in investigations and their applications are even more diverse. No single scientist can conduct all of the research involving a particular microbe or genetic material, such as a gene or plasmid. Scientific and technological progress, especially in molecular biology, bioinformatics, and nanotechnology, are providing researchers tools and knowledge for microbial investigations. All of these achievements are documented in various forms, such as publications and patents. This knowledge is not only used for conducting more effective and economic research but also for preventing a user from having to "reinvent the wheel" or invest considerable time and money in finding microbial genetic resources and their characterizations. To make mBRCs accessible to the microbiological research community and industrial scientists, dispatching microbial cultures is needed in compliance with national and international regulations.

Repositories of Microbial Genetic Resources and Receiving Safe Deposits for Publication and Intellectual Property Protection

The demand for microbes as starting genetic materials for research and applications is increasing annually. As microbial gene pools, mBRCs must have large quantities of diverse cultures. As custodians of national microbial resources, mBRCs must collect and accept deposits of microbes from internal and external sources. Deposits of microbes for the purposes of patent application and publication should be in compliance with the Budapest Treaty and a Material Transferring Agreement (MTA) with the depositor. In accordance with the Budapest Treaty, International Depositary Authorities keep deposited microbes secret but must furnish the microbes to entitled third parties upon application to the national patent authorities. Some culture collections also provide a special service for the long-term preservation of microbes. The distribution of preserved microbes may be restricted at the discretion of the depositor. Such "safe deposits" of microbes are a way to ensure long-term preservation without loss of ownership.

Conducting Research and Development (R&D) on Microbes

It is required for mBRCs to provide users with qualified microbes and related information. The quality of the microbes includes viability, purity, and identity. The required information mainly is the scientific name, source of isolation, and

conditions for handling and re-culturing. In addition, information on the microbe's biological criteria should be provided to acquaint the user with how to conduct appropriate research and applications. Thus, mBRCs can promote related research and disseminate expertise for preservation. Additionally, they maintain and distribute information related to taxonomy and identification, including the characterization of the most important biological criteria, biosafety, and application potential. The mBRCs maintain many microbes requiring them to balance these activities with other services.

Performing Related Service and Consulting
Based on R&D activities using microbes, mBRCs should be proficient in various techniques and methods and have experience in microbial preservation, taxonomy, identification, and other analyses related to the following activities (Çaktü and Türkoğlu 2011):

1. To provide identification services regarding different kinds of microorganisms in accordance with the expertise of the culture collection.
2. To organize training courses and workshops that are related to the identification and maintenance of microorganisms. Short courses and workshops are essential to train personnel from medical, environmental, industrial, and governmental laboratories that are responsible for isolating and identifying microorganisms, diagnosing disease, quality control, and fermentation, and culture management.
3. To provide general advice in the field of microbiology. This advice is not limited to research and the applications of microbes but includes consulting on internal and external policies and regulations relating to microbial sources.

3.2.2 Requirements for Establishment

The increasing demands on culture collections for authenticated, reliable biological materials and associated information parallel the growth of biotechnology. Recently, worldwide recognition of the necessity to conserve the microbial gene pool for future study and exploitation by mankind has highlighted the need for centers with expertise in cultural isolation, maintenance, identification, and taxonomy. Soon after the Earth Summit in Rio de Janeiro in 1992 approved the Convention on Biological Diversity (CBD) as an organization that promotes the culture collection of microorganisms, the WFCC prepared the first guidelines for the establishment and operation of such activities in 1994 (http://www.wfcc.info/index.php/wfcc_library/guideline/). The guidelines were improved in second (1999) and third (2010) editions to provide a framework for the establishment, operation, and long-term support of microbiological and cell resource centers as fundamental parts of the scientific infrastructure (http://www.wfcc.info/guidelines/). There are several key components that should

be taken into account for the establishments, such as (1) organization, (2) the aims or objectives, (3) funding sources, (4) holdings, and (5) human resources.

3.2.2.1 Organization

There is a parental body, under which mBRCs are established. The mBRCs should have the commitment from the organization for the long-term maintenance and implementation of its services. The activities of mBRCs should be appropriate for the organization's strategic plans or objectives. The organization may be classified into one of the two categories, government or nongovernment.

The mBRCs of universities and institutions, which are supported by the government, are the responsible authorities likely house public collections. These consist of a large number of strains and are established for the purpose of public service. The number of cultures varies from collection to collection. For example, the CBS (www.cbs.knaw.nl) in the Netherlands maintains over 18,000 strains of fungi, while there are 38,000 strains in the National Institute of Technology and Evaluation's Biological Resource Center (NBRC), Japan, and 37,000 strains in Thailand Bioresource Research Center of the National Center for Genetic Engineering and Biotechnology, Thailand.

For nongovernment sectors or private organizations, private or in-house culture collections are usually maintained by individuals, laboratories, institutes, hospitals, and commercial firms. The number of holdings may range from small (hundreds of strains) to large (thousands of strains). These culture collections can be used as in-house reference materials or be commercially and publicly accessible. Some collections are comparable to public collections, such as the American Type Culture Collection (ATCC), USA, with over 75,000 cultures. The strains in private culture collections are usually not available to the public.

3.2.2.2 Objectives

The mBRCs should have long-term objectives. These objectives should be related to the scope of the holdings and other envisaged services. In addition, some specific short- and midterm objectives are acceptable and helpful. However, the aims of mBRCs require careful consideration by, and appropriate to, the funding body. The objectives have to be practical and based on funding sources, holding, and available capacities, as well as manpower and facilities. Thus, the service and quality levels at various mBRCs are different. Some mBRCs focus on maintaining particular microbes, such as algae (Plymouth Algal Culture Collection, UK) or fungi (CBS, the Netherlands). The services of some private culture collections only include the preservation and distribution of microbial materials. In general, as standard roles of mBRCs, public mBRCs should provide important high-quality preservation: depository, distribution, taxonomy, and identification services.

3.2.2.3 Funding Sources and Facilities

Funding

Microbial domain Biological Resource Center (mBRC) facilities are ex situ, requiring long-term maintenance and related services. The parental organization must commit to funding the mBRC for the long term to maintain all its activities. Because the funding is crucial for quality and qualified mBRC-related services, the objectives and the activities have to be restricted. To match user's demands on cultures, many kinds of activities and services should be well planned with appropriate funding sources. In general, funding comes from two sources, the parental organization and service-associated fees or both. The parental organization is the government, private sector, or industrial support. Approximately 300 of 746 culture collections are supported by governments (http:/www.wfcc.info/ccinfo/statistics/). For most public mBRCs, the main funding (70–90%) is from the government, while services cover 10–30% of the total running cost. The funding required for developing countries is generally higher than those of developed countries. For many private mBRCs, the running costs come from the parental organization and services, but in some cases, they are partly funded by the government, which can subsidize private mBRCs that belong to quasi-government sectors, such as universities and institutions.

Facilities

Housing, laboratories, and equipment are important for quality control in mBRCs. The differences in holdings and servicing activities in various mBRCs result in differences in facilities. Progress in many fields of science and technology, including molecular biology, bioinformatics, nanobiotechnology, chromatography, and microscopy, has provided excellent tools for important service activities such as taxonomy, identification, and research. The users are in need of qualified microbial materials with well-documented profiles. Many standard mBRCs act as a unique research institutes in microbiology equipped with scanning electronic microscopes, transmission electronic microscopes, next-generation sequencers, matrix-assisted laser desorption/ionization in time-of-flight equipment, and gas chromatography–mass spectrometers. All of the equipment should be regularly maintained to ensure reproducible results.

3.2.2.4 Holdings

Holdings represent the scope and number of microbial genetic resources to be maintained. The holdings are required to match the objectives and the parental organization. Some mBRCs are specific to a particular group of microbes such as fungi, bacteria, algae, or microbes pathogenic to humans, animals, or plants. However, most of the public mBRCs such as the Vietnam Type Culture Collection (VTCC), Vietnam; ATCC, USA; Thailand Bioresource Research Center, Thailand; and NBRC, Japan, hold various kinds of microbes. There is no culture collection that can hold all microbes. To increase the effectiveness of the services

provided to the users, the storage capacities, human and financial resources, and other facilities have to be taken into account with careful consideration. To improve services for users, collaborations with other collections in a network at the national (Thailand Network Culture Collection), regional (Asian Consortium Microbial Resources), and global (World Federation for Culture Collections) levels are recommended. One important criterion for culture collections lies in reflecting the diversity of the local and national genetic sources to be maintained. To meet broad user demands related to fundamental and applicable studies, three kinds of cultures, type cultures, reference strains, and patent cultures are preserved. Duplicating cultures from other collections is not recommended. It is economically prudent to maintain complementary rather than duplicate resources; however, some duplication of important reference strains is acceptable. The WFCC recommended that new collections should collectively enrich the available genetic resources rather than duplicate those already existing; however, some authenticated internationally recognized reference strains are acceptable. The strength of a collection is indicated by the quantity and quality of distributed strains and provided services for a broad range of internal and external users. Thus, the diversity of maintained geographical microbes at country and region levels is crucial.

Microorganisms are useful and beneficial for mankind but can be misused against humans. For holdings of microbes pathogenic to man, animals, and plants, or those producing toxic compounds, biosafety and security must be maintained. This is a shared responsibility of government, science, industry, and the community, and they must work together to implement the best practices without impeding scientific development (Smith et al. 2017a). The holdings must be in compliance with all safety and security regulations in force, such as BTWC (Toxin Weapons Convention) or the EC Directive 2000/54/EEC on the control of biological agents. To develop safe practices, it is recommended to follow the guide in the OECD Best Practices on Biosecurity (2007) or World Health Organization (2006) on biorisk management (http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2006_6.pdf). National legislation in many countries requires permits or licenses to store, work with, and distribute such microbes. Unfortunately, many countries lack national regulations to manage biorisks (Smith et al. 2017b).

3.2.2.5 Staff

Managing a culture collection is a demanding task. It requires knowledge of not only the organisms themselves but of many other requirements, such as preservation, taxonomy, properties, and potential applications, including other services to meet the customer's requirements. The staff recruited would be expected to have culture collection curation skills.

The number of staff required for full- and part-time positions depends on the holding size and servicing activities, such as accession, preservation, quality control, and other services that the collection is required to provide. Unlike other positions, culture collection management involves repeated activities and requires reproducible

results. Thus, particular attention should be paid to the qualifications and experience of the staff in charge of the collection. To maintain the standards and quality of the culture collection and the services provided, retaining sufficient experienced and responsible staff is important. Frequent staff turnover is not recommended. Users of microbial materials are not always experts in microbiology; thus, they often erroneously handle the materials, fail to revive the culture, or contaminate the materials, resulting in claims against the service. In these cases, the curators have to address the claims. Thus, curators must have a wide knowledge of microbiology, not just in taxonomy but also in various related fields, such as physiology, biochemistry, pathology, and molecular biology. Curators should not have other positions or jobs. It is not possible for the culture collection staff to all be specialists in taxonomy, identification, and authentication; however, some basic taxonomic skills are needed for quality control. When in need of a specialists' expertise, such as in taxonomy or identification, it may be preferable to have collaborative arrangements with specialists outside of the organization.

3.3 Management and Operation

The general service collection of microorganisms is part of the essential infrastructure for the life sciences in which microorganisms and their derived materials are employed in both fundamental and applied research activities for various fields, such as biotechnology, industry, agriculture, medicine, pharmacy, and environment. The crucial functions of culture collections are preserving, supplying, and collecting microbial sources.

3.3.1 Preservation

The objective of preserving and storing cultures is to maintain them in viable states without morphological, physiological, or genetic changes until they are required for use. Ideally, microorganisms require special preservation methods to ensure the maintenance of the following crucial indicators:

- Optimal viability/recovery of the preserved culture. Cell death may occur during the preservation process and further losses during storage. To avoid the loss of cells, the preservation process and methods used should minimize the loss of viability over a long period.
- Purity. No contamination should be present in the preserved culture (this does not include any recognized co-culture, such as symbiotic microorganisms or microbial communities, which are not regarded as contaminants). Cultures preserved for both fundamental and applied uses are required to be pure to provide

reproducible results. The preservation method used should minimize the probability of contamination.
- Stability. The preserved culture is valuable and important for various uses in the life sciences and industry. During the preservation process and storage, some mutations may occur or plasmids lost, resulting in the loss or gain of some characteristics. Thus, the preservation method should minimize undesirable changes in morphology, biochemical properties, genetic profiles, immunity levels, and pathogenicity.
- Identity/authenticity. Preserved cultures should be the same as the original culture deposited at the mBRC by the depositor.
- Long-term security. The cultures should be stored using at least two different preservation methods. One should be a long-term preservation method, either lyophilization or quick freezing in liquid nitrogen. It is recommended that a backup collection should be stored at a distant site to protect the cultures from any disaster.

There is a wide variety of available methods for the preservation of microorganisms. The method chosen for preservation depends on some crucial factors, as listed below (Kirsop and Snell 1984):

- *Funding:* Maintaining culture includes the cost of staff, equipment, materials, and facilities, such as laboratories, storage space, and power supply.
- *Holdings*: The number of cultures maintained and the kind of microorganisms may require particular methods, staff skills, equipment, and storage space.
- *Value of the culture:* The consequences of the loss of a culture should be considered when choosing one preservation method.
- *Supplying and transporting cultures:* If the cultures are to be distributed, then replicates are needed. For later distributions, cultures should be prepared in bulk and stored. The convenient distribution of cultures depends on the preservation method used and the number of cultures to be distributed. If cultures are to be supplied by mail, then they must be packaged in a suitable form to survive any possible delay in delivery.
- *Frequency of culture use*: Some cultures are frequently used as assay strains or in industrial production. In these cases, the risk of contamination is high, so the use of stock cultures should be considered.

It may be difficult to choose the most appropriate method for a particular microorganism. For culture of each microorganism, an appropriate method (s) should be chosen by the mBRC, the recommendation of the depositor, or some other reference mBRC, such as the NBRC (Japan), the ATCC (USA), and CABI (England). The available methods range from growing the culture at a reduced metabolism rate to the ideal situation in which the metabolism is suspended. The important available preservation methods may be classified into three categories, sub-culturing, drying, and freezing.

3.3.2 Sub-culturing Preservation Methods

Sub-culturing This is one of the oldest and the most traditional methods of preservation. The microorganism is grown on appropriate medium over a period of days or weeks. Then, the culture is transferred to storage at a low temperature, normally 5 °C for a variety of microorganisms. However, not all microorganisms survive longer at low temperatures. For example, *Neisseria* spp. survives better at 37 °C (Kraus 1979). The process is repeated at an appropriate interval that ensures the preparation of a fresh culture before the old one dies. The period of preservation is different depending on the culture. Many bacteria, yeast, and microalgae strains can survive from some months to years. The advantage is that the method is inexpensive in terms of equipment and little skill is required. Sub-culturing is the simplest and most common method used in small laboratories, especially if cultures are required frequently and quickly. In addition, this method is applicable to a wide range of microorganisms. However, this method has some disadvantages. The most notable disadvantages are:

Contamination: This occurs frequently, especially when large numbers of cultures are involved or the technician is distracted.
Change in characteristics: Sequential transfers often result in high rates of mutation and plasmid loss. The culture's characteristics, such as enzyme activity, immunity, and pathogenicity, are not stable, and undesirable characteristics may arise.
Mislabeling and misplacement: Cultures may be labeled with the wrong name or number. The label may become distorted or illegible. Wrongly inoculated cultures may occur when a large number of cultures are being transferred. Cultures may be inoculated into the wrong tube or several organisms placed in the same tube.
Culture loss: In many cases, the loss is related to equipment failure, such as insufficient stability in incubation and refrigeration temperature.
Storage space: Large numbers of test tube cultures require very large storage and incubation spaces.

3.3.2.1 Filamentous Fungi

1. *On agar medium* Fungi can be grown in various common agar media, such as Malt Agar, Czapek Dox, potato dextrose agar (PDA), yeast extract sucrose, and Sabouraud dextrose agar. However, some fungi require a specific medium, such as *Corticium praticola*, which requires soil-extract agar. The culture is grown in the medium under optimum conditions, including temperature, aeration, pH, and humidity. It is important that the pure culture is in good health prior to use as an inoculum with the full morphological and bio-physiological criteria as the original deposit.

Using spores from a culture as inoculum is optimum, but mycelia of sporeless fungi are acceptable. It is recommended that the culture be undergoing sporulation or

growth before storing. In general, the storage temperature is lower, slowing the metabolic rate, which allows more time between sub-culturing. Storage at 4–7 °C or in a cold room can extend the transfer interval to 4–12 months. Cultures growing on agar may be infested by mites. Mites can be detected by the naked eyes at twice weekly intervals. If mites are detected, then the contaminated culture should be destroyed by autoclaving at 121 °C for 15 min (Hunter-Cevera 1996).

To increase the interval transfer, cultures can be stored under oil or water:

2. *For storing under oil* This method is the simplest and most inexpensive for long-term culture preservation, especially for fungi that do not tolerate freeze-drying (Humber 1997). The oil prevents desiccation and diminishes gas exchange, resulting in a reduced fungal metabolism. Cultures kept under mineral oil may remain viable for decades (Mendes da Silva et al. 1994). Some cultures, such as those of *Aspergillus*, *Penicillium*, *Phytophthora*, and *Pythium*, in tubes/bottles with loosened caps can be stored at 15–20 °C for 1–40 years. However, for other organisms such as *Cercospora*, *Arthrobotrys*, *Colletotrichum*, *Conidiobolus*, *Corticium*, *Nodulisporium*, and Basidiomycetes cultures should be transferred at 2-year intervals (Hunter-Cevera 1996). To store fungal cultures under oil, the mature culture is placed on an agar slant or aseptically covered bottle of liquid paraffin [medicinal paraffin, specific gravity of 0.830–0.890 to a depth of 1 cm (Kirsop and Snell 1984)], which underwent autoclaving (121 °C, 15 min) twice, 24 h apart. The culture is covered with a tight cap and stored upright in a rack at 15–20 °C. A sterile loop or needle is used to recover an explant from the culture. Drain the excess oil before placing the sample on fresh appropriate agar petri plate medium. The culture must be monitored for viability and contamination before transferring to a fresh slant agar medium to obtain a pure culture.

3. *Storing under water* The storage of metabolically inactive fungi under sterile distilled water may be the least technologically demanding of any preservation techniques. This method has been used successfully with a wide range of fungi, including human and plant pathogens, and the *Entomophthorales*. The viability levels of some fungi are different from individual cultures. A shelf life of 20 years has been documented with this method (Castellani et al. 1967; Figueiredo and Pimentel 1975). However, it is recommended that cultures be stored for 2–5-year periods (Hunter-Cevera 1996). To recover the culture, the same techniques and attention as described for oil storage are applicable.

3.3.2.2 Mushrooms

A mushroom (or toadstool) is the fleshy, spore-bearing fruiting body of a fungus, typically produced above the ground on soil or its food source. The standard "mushroom" is the cultivated white button mushroom, *Agaricus bisporus*. Thus, the word "mushroom" is most often applied to those fungi (*Basidiomycota* and *Agaricomycetes*) that have a stem (stipe), a cap (pileus), and gills (lamellae) on the

underside of the cap. Mushrooms have significant values. Edible mushrooms such as *Agaricus* and *Pleurotus* are useful food sources and with some, such as *Ganoderma*, *Lentinula edodes* (shiitake), and *Grifola frondosa* (maitake), being good sources of functional foods or traditional medicinal materials, although many are toxic to humans and animals, such as *Amanita*, *Cortinarius*, and *Podostroma.*

Mushrooms are invariably stored as mycelial cultures because the spores of heterothallic and secondary homothallic species are produced through asexual processes and have genetic differences, which may eventually result in the failure to fruit. In the absence of hardy structures, like double-walled spores or sclerotic, cultures become susceptible to sudden changes in temperature and pressure.

Sub-culturing is used for current applications of many genera. The techniques are simple and much the same as for filamentous fungi. In our laboratory (VTCC), cultures are sub-cultured onto complete PDA medium slants. The cultures are incubated at 28–30 °C for 7–14 days to achieve full mycelial growth before storing at 5 °C. Some strains prefer to be stored at 10–15 °C. The shelf life is 3–6 months, depending on the individual strain. Using this method, the shelf life of *Lentinula*, *Pleurotus*, and *Agaricus* were up to 6 months (Chang and Miles 2004). Additionally, various mushroom cultures have been stored on sorghum (jowar) grains at low temperatures of 5–8 °C in a refrigerator (Veena and Pandey 2010). The mushroom cultures could be safely stored at low temperatures on sorghum grain free from contamination for longer than 1 year without any growth or morphological changes. The most significant advantage of this method is its ability to conserve milky mushroom (*Calocybe indica*) and some isolates of reishi mushroom (*Ganoderma lucidum*) cultures, which cannot be stored at low temperatures. Recently, Garcia-Garcia et al. (2014) reported a filter paper discs method for storing medicinal mushroom mycelia in sterile distilled water (Fig. 3.1). *Humphreya coffeata* (Berk.) Stey. stored at 4 °C was viable for up to 18 months, with no visible morphological alterations or contamination by bacteria or other fungi. Thus, this method, in addition to being easy and economical, is suitable for the conservation of higher Basidiomycetes. It should be noted that the viability period depends on the fungal species being stored. The method is briefly described below:

Fig. 3.1 Scheme of the filter paper disc method for mushroom preservation

1. Preparing filter paper discs and agar petri medium: Sterile petri dishes (110 × 25 mm) are aseptically filled with 30 ml suitable medium, such as malt extract agar. Filter paper discs with 5 mm diameters (Whatman No. 4) are manually prepared and sterilized.
2. Preparing the mushroom mycelium: The central area of the prepared petri dishes is inoculated with the culture, and 6–8 sterile prepared filter paper discs are then placed around the inoculum point. The petri dishes are incubated at 30 °C for 4–6 days.
3. Storing the mycelial discs: After the culture grows well, mycelia cover the petri dishes and filter paper discs. Using sterile small forceps, the paper discs with mycelia are removed and aseptically placed in vials with sterile screw caps containing 4 ml of sterile distilled water. All of the vials are closed and sealed with Parafilm. Finally, the vials are stored at 4 °C.

   Cultures are recovered by aseptically removing the culture disc and placing it onto a petri dish containing an appropriate medium.

3.3.2.3 Mycorrhizae

Mycorrhizae are symbiotic associations between fungi and the roots of a vascular host plant. Mycorrhizae are commonly divided into *ectomycorrhizae* (ECMs) and *endomycorrhizae* or arbuscular mycorrhizal fungi (AMFs), arbuscular mycorrhizas (AMs), and vesicular-arbuscular mycorrhizae (VAMs). AMFs are a group of obligate biotrophs to the extent that they must develop a close symbiotic association with the roots of a living host plant to grow and complete their life cycle. The hyphae of endomycorrhizal fungi penetrate the cell wall and invaginate the cell membrane. AMFs can symbiotically interact with almost all plants that live on the Earth. They are found in the roots of ~80–90% of plant species (mainly grasses, agricultural crops, and herbs) and exchange benefits with their partners, as is typical of mutual symbiotic relationships. They represent an interface between plants and soil, growing their mycelia both inside and outside of the plant roots. AMFs provide the plant with water, soil mineral nutrients (mainly P and N), and pathogen protection. Spores are produced at the tips of hyphae either within the host root or outside the root in the soil as propagules. These spores germinate, and the germination tube that is produced grows through the soil until it comes into contact with roots. The fungus then penetrates the root and grows between root cells, or it may penetrate the cell wall and grow within root cells. Inside the root, the fungus forms arbuscules, which are highly branched hyphal structures that serve as sites of nutrient exchange with the plant. However, the hyphae of ECMs do not penetrate individual cells within the root. Their dominant form in vitro is hyphae that generally grow slowly in axenic cultures (Molina et al. 2003). Hyphae are usually more sensitive to environmental conditions than spores or conidia (Smith 1993) because of their characteristics (i.e., the absence of conidia and few or no spores produced in vitro).

- ***AMFs***: Maintenance/preservation is mainly through sub-cultivation, either in vitro on a synthetic growth medium, in association with transformed roots, or in vivo in association with (host) plants in the greenhouse. The storage methodologies should preserve a product's high and consistent quality and be simple and inexpensive. AMF viability and efficiency can be maintained for several months at room temperature (20–25 °C), but the inocula must be kept in their packaging and must be partially dried. The main inconvenience during the storage period is that spores can sometimes become dormant, decreasing the germination rates drastically (Oehl et al. 2009). However, a cold-storage period can be used to break dormancy (Juge et al. 2002).
- ***ECMs***: The sub-culturing methods used for mushrooms are applicable. The time between sub-cultures can be extended by reducing the storage temperature. For instance, Tibbett et al. (1999) stored a wide geographical range of *Hebeloma* isolates for 3 years at 2 °C. Additionally, the survival of ECM fungi stored in sterile water at 5 °C was 100% after 1 year but declines for some isolates to 95 and 64% after 2 and 3 years of storage, respectively (Marx and Daniel 1976). Thus, this method is only considered useful for short-term preservation (2–5 years). Maintenance occurs through sub-cultivation at room temperature or preferably under refrigeration. Storing ECM mycelia under water or oil is an easy to apply method. These methods are adequate to maintain some ECM fungi for periods of up to 20 years (Richter 2008).

3.3.2.4 Yeast

Sub-culturing yeast has been using successfully for many years for short-term preservation. The long-term preservation for maintaining yeast is reported by NCYC (National Collection of Yeast Cultures) (Smith et al. 2001). Sub-culturing method can be used for both liquid and solid media. In this method, an inoculum is regularly transferred to a fresh media. All of the yeasts maintained by these methods show high recovery rates (Smith et al. 2001). None of the fermentative strains survive better on agar slants than in broth. Approximately 10% of strains show changes in some characteristics, such as flocculation behavior or other morphological and physiological properties, after long periods, such as 10 years (Kirsop and Snell 1984).

Sub-culturing in Broth Medium

Preparation: Dispense 10 ml medium yeast extract and malt (YM) broth (Difco 071101) into McCartney bottles. Sterilize by autoclaving at 121 °C for 15 min.

Inoculum: Transfer a loop of stock culture aseptically to the prepared McCartney bottle.

Incubation: Incubate the inoculated bottle with shaking at 25 °C for 72 h. Examine visually for growth at 72 h. If the growth is poor, then more incubation time is needed. Examine daily. In some cases, it is necessary to aerate the culture by

shaking (for some aerobic genera, such as *Cryptococcus*, *Rhodotorula*, and *Sporobolomyces*).

Storage and shelf life: The common storage temperature is 4 °C. In general, the culture will remain viable under these conditions for 6 months. Fermentative species survive better than non-fermentative species, and some of the latter may need to be sub-cultured more frequently, perhaps at 2-month intervals.

To prepare duplicate cultures, one is for all operations and the other to act as the new stock culture.

Sub-culturing on Agar Slants

In total, 10 ml YM broth (Difco 071102) is dispensed into screw-cap loosened McCartney bottles. Then, the samples are sterilized by autoclaving at 121 °C for 15 min and allowed to sit at an inclined angle to form slants. All of the sub-culturing steps in broth are applicable to this method. The shelf life of many yeast strains will be longer on agar slants than in broth, particularly the non-fermentative genera. However, ascosporogenous strains that sporulate on the agar slants will likely become unstable. If this characteristic is a priority, then these methods should not be used.

Sub-culturing on Agar Slants with an Oil Overlay

In this method, before storing at 4 °C, the culture on the agar slant is overlaid aseptically with sterile oil (B.P. medicinal oil; BDH Chemical Ltd.) to 1 cm above the top of the agar slant. The shelf life is then extended from 2 to 3 years. Care must be taken during sub-culturing not to let the inoculation loop splutter when flaming. Pathogens should not be maintained using this method.

3.3.2.5 Bacteria and Actinomycetes

In general, sub-culturing on agar slants (or petri dishes) and bottles is applicable for bacteria. Because bacteria are very diverse, each organism needs to be grown on a suitable medium under selective conditions. There are hundreds of media for bacteria such as Luria-Bertani, MRS, yeast–glucose agar medium, and sporulation medium for *Streptomyces*. The interval for sub-culture preservation of bacteria on agar media is approximately 1 month, and the period for broth sub-cultures is shorter. Agar petri plates are the preferred media for sub-culture preservation. On plates, the cultures are more easily verified by comparing with a pure culture that shows a type of colony or to see if contaminating colonies arise. In addition, if a plate is contaminated, then it should be discarded. However, in many cases, the target colony can be isolated with a sterile transfer loop and streaked to a fresh agar plate for recovery. For actinomycetes, sub-culturing to agar slants is performed much as it is with fungi rather than with bacteria. Actinomycetes need longer culturing and spore forming periods (10–14 days). For anaerobic bacteria, an agar slope medium can be used, such as MM10 (Caldwell and Bryant 1966) medium. The medium is inoculated by stabbing and then incubating at room temperature in the dark for ~4 weeks. Sub-culturing in broth having a low glucose (BGP or BGPhlf) level is also

applicable. The culture broth should be stored at room temperature in the dark with the caps screwed down tightly. The shelf life is ~1 year, and in the special case of the spore-forming anaerobic bacteria, *Clostridia* can survive for several years. The technique is described in item 2.1.2.

3.3.2.6 Algae

When handling many algae strains, practical considerations should be maintained with respect to individual optimum conditions and media. Individual strain shows diverse requirements for media, light, pH, and temperature. Nutrition for algae ranges from obligate phototrophy to heterotrophy. Nutritional requirements vary from strain to strain. Thus, extensive trials are required to determine the best medium for an individual strain. For healthy growth, many strains require extracts from soil, plants, or seawater in the medium.

Sub-culturing on liquid and agar slants is commonly used for short-term preservation. The shelf life is 2–3 months for liquid and 1–6 months for agar slant preservation (Kirsop and Snell 1984). For the latter, the plugs or caps should not be too tight because a gaseous exchange with the external environment is required.

3.3.3 Drying Preservation Methods

The principle of drying methods is the removal of water from the culture, resulting in the suspension of cell metabolism. There are a number of drying methods for culture preservation that are used for various microorganisms, such as on soil/sand, glass beads, silica gels, freeze-drying (lyophilization), and L-drying (liquid drying) (Fig. 3.2). Sodelli's method of preservation is simpler than the lyophilization method that is described by Sharma et al. (2017a). The methods vary from having a high cost and requiring skill, such as for lyophilization and L-drying, to having a low cost and requiring limited skill.

3.3.3.1 Filamentous Fungi

Storing on Silica Gel

Silica gel has been successfully used to maintain diverse species of fungi. It is inexpensive and requires limited technical experience. Fungi can be stored for 7–19 years when covered well and remain morphologically and genetically stable. Briefly, glass bottles are partially (one-third) filled with silica gel and sterilized in an oven (180 °C for 3 h). The bottles are placed in a tray of water, which is placed in a deep freeze (~−20 °C) overnight. Then, the tray is removed from the deep freeze 20–30 min before use. Spore suspension is made in 5 °C sterile 5% (w/v) skimmed milk and added to the silica gel (up to three-quarter of the silica gel's volume). The

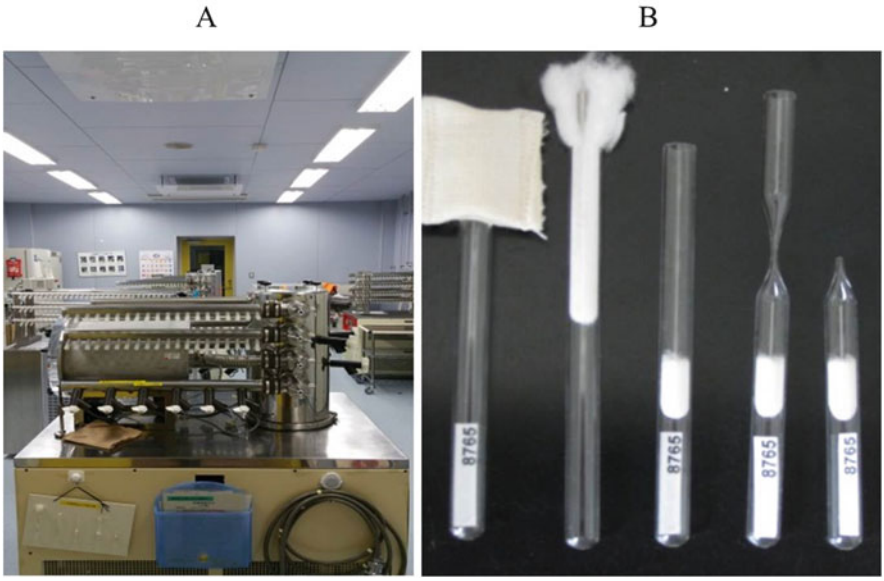


Fig. 3.2 (**a**) Lyophilizer (NBRC, Japan) and (**b**) lyophilized ampoules (Peter Green, National Collection of Industrial Food and Marine Bacteria Ltd.)

bottles are shaken to agitate, coating spores with silica gel. After incubating at 25 °C, when the silica gel is readily separated, the bottles are tightly capped and stored at 4 °C in airtight containers. Recovery is simple. A few silica gel-covered spores are sprinkled on a suitable growth medium and incubated under appropriate conditions (Smith et al. 2001). However, this method is not recommended for fungi with thin-walled spores or spores with appendages or mycelia, for example, the zoospores and oogonia of Oomycetes, such as *Phytophthora*, *Pythium*, *Bartalinia*, and *Cercospora* species (Kirsop and Snell 1984).

Soil/Sand Storage

Preservation in soil/sand is a practical and cost-efficient way to store filamentous sporulating fungi. Preserved cultures remain viable for up to 10 years, and the method reduces the occurrence of mites. Several fungi can be preserved using this method, such as *Fusarium* (Gordon 1952), *Rhizopus*, *Alternaria*, *Aspergillus*, *Circinella*, and *Penicillium* (Atkinson 1953). Soil/sand storage can be used for some fungi that are not stable under oil.

Briefly, a mound of garden soil/sand, with about 20% moisture, is placed into glass bottles at two-thirds capacity. The bottles are sterilized twice, 24 h apart, at 121 °C for 15 min. Then, 1 ml of spore suspension was added in sterile water to the bottles with loose caps and kept at room temperature for 5–10 days to grow and dry. For strains that do not sporulate, a mycelial suspension can be used. Finally, the bottles are stored at 4–7 °C. Recovery is simple. A few grains of soil/sand are

sprinkled on a suitable growth medium and incubated under the appropriate conditions.

Lyophilization/Freeze-Drying Method

This method is highly successful for preserving sporulating filamentous fungi. During the freeze-drying process, water is removed directly from frozen materials by sublimation under a vacuum. If carried out correctly, freeze-drying will prevent shrinkage and structural changes and help retain viability. There is a vast array of freeze-drying equipment available, ranging from laboratory bench models to huge industrial installations. This method can be optimized for different organisms or cells.

The freeze-drying method has many advantages over other methods. Generally, preserved cultures have good viability and stability and can be stored for many years. Many genera, including *Almaria*, *Aspergillus*, *Botryotrichum*, *Cephalosporium*, *Cladosporium*, *Cunninghamella*, *Chaetomium*, *Cordyceps*, *Fusarium*, *Gilberella*, *Mucor*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Rhizopus*, and *Trichoderma*, can survive for 14 years (Kirsop and Snell 1984). Storing ampoules requires little space, and they are easily distributed through the mail. However, the method has some disadvantages, such as requiring expensive equipment and having a complex protocol that necessitates a skilled time-consuming technique. Some genera that fail to survive include *Ganoderma*, *Entomophthora*, *Phytophthora*, and *Pythium*. Reduced viability and genetic changes may occur as reported by Ryan et al. (2003).

Some main factors that affect the viability and stability of the culture are as follows:

1. The suspension medium should be convenient to use and protect the spores from freezing- and storage-related damage. The components are skim milk, serum, various kinds of sugars, and peptone.
2. The freezing rate is a very important factor that must be considered to obtain the optimum recovery rate. A slow freezing rate (−1 °C/min) has been successfully applied to many strains. The technique of centrifugal freeze-drying relies on evaporative freezing under reduced pressure, which results in a favorable cooling rate and can be used successfully for many sporulating fungi (Smith 1993).
3. Sealing the ampoules is the most important factor for preventing the deterioration of freeze-dried cultures.
4. The moisture content affects the stability of organisms. To achieve the best recovery, a 5% moisture content should be reached before the sample's temperature rises above −15 °C. Overdrying will kill or cause mutations by damaging DNA; thus, the residual moisture content must not be allowed to fall below 1% (Sneel 1984).
5. Ampoules of freeze-dried samples must be stored out of direct sunlight to avoid shortening the shelf life.

   The centrifugal freeze-drying method (Smith et al. 2001) is briefly described as follows:

The spore suspension is prepared in a mixture of a 10% (w/v) skim milk and 5% (w/v) inositol. The spore suspension aliquots of 0.2 ml are added to each sterile

ampoule, covered with a sterile cap. Load the ampoules into a spin freezer, and place it in the dryer. Spin for 30 min, and cool to −40 °C. Evacuate the ampoules in the chamber for 3 h until the moisture content of the sample would be around 5%. Remove the ampoules, and replace the cape by a sterile cottonwood plug; compress the tin at 10 mm depth and 10 mm above the sample. Constrict the plugged ampoules at 10 mm above the plug using a gas torch at about 1 mm. Place the constricted ampoules on the secondary-drying accessory of the freeze dryer, and evacuate over a phosphorous pentoxide desiccant for approximately 17 h. The ampoules are sealed at the constricting point using a crossfire burner under a vacuum. Maintain the ampoules at 4 °C.

To recover a sample from a stored ampoule, score the ampoule at the center of the cottonwood plug using a glass cutter, and then heat with a Bunsen burner until red hot, and apply pressure firmly to the score. Aseptically, open the ampoule, and remove the cottonwood plug, and then add 2–4 drops of sterile distilled water, and leave for 30 min to rehydrate the suspension. Finally, inoculate the suspension onto a suitable growth medium, incubating under appropriate conditions. Check the growth and viability.

3.3.3.2 Mushrooms

The lyophilization method is preferred for the long-term preservation of spore-bearing mushrooms. It is not useful for storing the mycelia of mushrooms. The survival rates of freeze-dried fungal hyphae of a number of ascomycetes have been recorded as being higher than two Basidiomycetes, *Schizophyllum commune* and *Coronus psychromorbidus*. The protocol for storing spore-bearing mushrooms is almost the same as for fungi. However, Singh and co-workers (2004) developed a method for the preservation of mushroom mycelial cultures. Briefly, all of the stock cultures are sub-cultured on pearl millet (*Pennisetum typhoides*) grains in test tubes containing wheat extract agar. All of the inoculated test tubes are then incubated at 25 °C for 14 days, except those of *Volvariella volvacea*, which should be incubated at 32 °C for 10 days. Mycelial cultures multiplied on pearl millet grains are subjected to lyophilization. For lyophilization, glass ampoules are first sterilized in a hot-air oven at 180 °C for 2.5 h and allowed to cool at ambient temperature. The mycelia that are multiplied on pearl millet grains are placed using sterilized forceps into a sterilized ampoule. Each ampoule containing 50–60 grains is plugged with sterilized nonabsorbent cotton. The cotton plugs are pushed inside up to the neck of the ampoules. The constrictions are made from above the cotton region in each ampoule and pre-cooled in a deep freezer to −40 °C. When the freezing chamber reaches −62 °C, the ampoules are attached to the lyophilizer and a vacuum created. The lyophilizer is run overnight. The following day, when the pressure reaches 0.05 mbar, the ampoules are sealed at the point of constriction with the help of a crossfire burner connected to oxygen and gas. The vacuum is tested using a vacuum tester. A purple-colored light inside the sealed ampoule verifies proper sealing. The prepared ampoules are stored at room temperature. For recovery, grains (pearl millet

seeds containing mycelia) are placed in pre-sterilized petri plates containing malt glucose agar.

3.3.3.3 Mycorrhizae

For ECMs: Because ECMs lack conidia and produce few or no spores, the freeze-drying method of preservation is not strongly recommended. However, this method is being used for nonspore-bearing cultures too. A *Laccaria fraterna* culture from the late to stationary phase of growth, treated with 10% dimethyl sulfoxide (DMSO) as a cryoprotectant, survived the freeze-drying method. The morphological and physiological characteristics were similar between the freeze-dried and nonfreeze-dried samples (Sundari and Adholeya 1999).

For AMFs: The advantage of AMFs over ECMs is that they are spore-producing fungi. Thus, the drying methods used for filamentous fungi (Smith 1993) can be applied to AMFs. AMFs are mostly cultured in vivo, but a limited number of species are cultured in vitro. Several preservation methods using propagules produced either in vivo or in vitro have been developed. For isolates produced in pots, drying the soil containing AMF propagules is generally achieved at room temperature, for 2–4 days before lyophilization. For isolates produced in vitro, the drying of the carrier (i.e., alginate beads containing AMF propagules) is crucial and should be preceded by incubation in a cryoprotectant (ideally trehalose) at 27 °C for 2 days (Lalaymia et al. 2014). Whether cultured in vivo or in vitro, it is recommended to use propagules from cultures in the late or stationary phase of growth. The preconditioning of AMF propagules for lyopreservation should be conducted in a carrier (i.e., soil or alginate beads) to protect them from preservation stresses and facilitate their manipulation. The carrier should be dried before lyopreservation to prevent water crystallization outside and inside the propagules. For in vitro-cultured AMF propagules, we recommend the use of 0.5 M trehalose as a cryoprotectant, while the in vivo-cultured propagules can be preserved in dried soil without a cryoprotectant.

3.3.3.4 Yeast

Commonly, there are three drying methods for yeast preservation, namely, silica gel, paper replica, and freeze-drying. There is no relationship between survival and taxonomic position, and the factors determining survival are specific to each strain. Thus, a method that is satisfactory for one strain may be unsuitable for others. Whichever method is selected, the most suitable growth conditions for the cells must be established. Both the age of the culture and the oxygen availability during growth can affect the survival rate. Thus, post-logarithmic cells usually survive better than younger cells (Kirsop and Snell 1984).

Silica Gel Storage
Fill McCartney bottles to a depth of 1 cm with grain plain 6–22 mesh non-indicating silica gel, and sterilize in an oven at 180 °C for 90 min. Cool down the bottles, and transfer to an ice tray. Aseptically add a few drops of the yeast suspension prepared in pre-cooled 5% (w/v) skim milk to each gel bottle. Return the inoculated bottles to the ice tray for an additional 30 min. Screw the cap on tightly, and maintain at room temperature for approximately 14 days. The culture is dry when the gel crystals separate. Transfer the culture to an airtight container with a layer of indicator silica gel in the bottom. Store at 4 °C. Check the indicator gel occasionally and redry by heating in an oven at 180 °C for 2 h if necessary. Recovery is the same as for filamentous fungi. Using this method, the shelf life is 2–3 years and up to 5 years as reported by Woods (1976).

Paper Replica Method
Kirsop and Kurtzman (1988) reported that yeast could be dried on filter paper and stored above desiccant silica gel. To prepare the culture for storage, squares of Whatman No. 4 filter paper (10 mm) are used. They are inoculated by an immersion in drops of yeast suspension previously prepared in 5% (w/v) skim milk. The inoculated filter paper squares are dried in a desiccator in the foil packets and stored in an airtight container at 4 °C. The cultures are recovered by removing the filter paper, placing the square on an appropriate agar plate medium, and incubating at a suitable temperature. The shelf life is ~2–3 years.

Freeze-Drying Method
The protocol of centrifugal freeze-drying method (Smith et al. 2001) for yeast is briefly described as follows:

1. Grow the culture without aeration in YM broth (Difco 0711-01) at 25 °C for 72 h.
2. Prepare the yeast suspension by mixing the grown culture and freeze-drying medium. Select one of the following media: (1) 20% skim milk and 10% glutamate; (2) 5% honey, 10% skim milk, and 10% dextran; and (3) 5% honey, 10% skim milk, and 5% glutamate at a culture concentration of at least 10^6 cell/ml.
3. Remove the cotton plugs, and aseptically add 0.2 ml of the cell suspension to each ampoule using a Pasteur pipette, following the steps as described in item 2.1.2.1 previously for filamentous fungi.

The recovery procedure is the same as that for filamentous fungi, except that an aliquot of YM broth from a Bijoux bottle containing 1 ml of sterile broth is added to the materials, and then the suspension is returned to the remaining YM broth in the Bijoux bottle before incubating under appropriate conditions. Check the growth and viability.

3.3.3.5 Bacteria and Actinomycetes

Common drying methods for the storage of bacteria and actinomycetes include gelatin disc, soil, lyophilization, and L-drying.

Gelatin Disc Method

The method is described in detail by Kirsop and Snell (1984):

1. Prepare a bacterial suspension of 0.5 ml in nutrient broth, and add to 3 ml of the gelatin suspension medium (10 g gelatin powder, 2.5 g nutrient broth powder, 5 g meso-inositol, and 100 ml deionized water) that was previously melted at 37 °C, pH 7.2. Place 3 ml aliquot in 6 ml bottles, and sterilize by autoclaving at 121 °C for 15 min.
2. With a pipette, dispense dropwise 0.02 ml of suspension to the base of a plastic petri dish. Approximately, 80 drops can be accommodated in the base.
3. Freeze the drops on the petri base by placing the petri dishes in a freezer (–20 to –40 °C for approximately 2 h).
4. Freeze-dry the drop by transferring the petri dishes quickly to the freeze dryer (which must be loaded with phosphorous pentoxide).
5. Freeze-dry the culture overnight. For a large number of discs in a batch, it may be necessary to replace the phosphorous pentoxide after 2–4 h.
6. Preparing the vials. Into each screw-necked vial, add self-indicating silica gel (to a depth of approximately 10 mm), and pack down tightly with cotton wool, cover with foil, and sterilize at 160 °C for 1 h. The caps are sterilized in an oven at 60–80 °C for 4 h before placing them on the vials.
7. Aseptically transfer the sterilized discs to the vials when the freeze-drying process is finished.
8. Replace the caps of the vials and tighten. Then, store the samples at 5 °C.
9. The shelf life of the method is ~3–4 years. After using this method for 4 years, the NCTC has found little change in phenotypic characteristics of strains used as controls or in identification kits.

   For recovery, select a gelatin disc with sterile fine-nosed forceps, and place it in a test tube containing 1 ml of nutrient broth. Warm the sample at 37 °C until the disc dissolves. A loop of the suspension broth is transferred to a suitable medium agar in a petri plate and streaked for single colonies before incubating at an appropriate temperature.

Soil Storage for Actinomycetes Preservation

The method is described in detail by Dietz and Currie (1996).

A loam soil is the most practical and commonly used for this method. The soil should not have been fertilized or treated with pesticides or herbicides. It is heated at 160 °C for 6 h to destroy spore-forming microorganisms or insects that may be present. The soil is sieved using a 30 mesh sieve. The sieved soil is added to Pyrex tubes (13 × 100 mm) to a depth of 20 mm. The soil tubes are autoclaved separately for 60 min each day for 3 days. It is necessary to check for the contamination of the sterilized tubes by adding a loopful of the soil as seeding to a suitable medium and incubate at 24, 28, and 37 °C. Check for contaminating microorganisms at the appropriate incubation times.

1. Prepare the culture for storage. Make the spore suspension by adding a suitable amount of distilled water to a sporulating culture.

2. Add 1 ml of the spore suspension to each tube.
3. Air-dry the culture tubes at room temperature (24 °C) for a month. Tap the culture tube to loosen the soil particles.
4. Store at 4 °C.
5. The shelf life of a culture stored using this method is 2–3 years. Better results may be achieved if a dried blood preparation (100 g soil, 10 g $CaCO_3$, and 2.5 g dried blood) is used.

For recovery, a sterile loop is moistened in sterile distilled water, and then the soil is aseptically picked up on the wet loop. Place the inoculum on a suitable medium, and incubate at an appropriate temperature.

Lyophilization (Freeze-Drying) Method

Lyophilization is a process in which water vapor is removed directly from frozen materials by sublimation. This method has been used for many years to preserve a wide variety of biological materials and bacterial strains. This is an expensive method but one of the best for the long-term maintenance of microorganisms. The below method is described by Kirsop and Snell (1984).

1. Prepare the culture suspension by adding 2–4 ml of one of the three possible sterile suspensions, (1) 100 ml horse serum, 33 ml Oxoid CM1, and 10 g glucose; (2) 5 g meso-inositol and 100 ml horse serum; or (3) 2.5 g nutrient broth powder No 2 (Oxoid), 5 g meso-inositol, and 100 ml distilled water, to an agar slant of the culture grown to late log or early stationary phase, and mix.
2. Aseptically add 0.2 ml of the cell suspension to each sterile ampoule using a sterile Pasteur pipette.
3. Replace the lint caps, and load the ampoules into the centrifuge plate of the freeze dryer, ensuring the plate is balanced.
4. Place the centrifuge plate in the chamber of the freeze dryer, and evacuate the chamber. After 20 min, switch off the centrifuge, and start the primary drying of at least 3 h.
5. Admit air into the freeze-dryer chamber, and remove the ampoules.
6. Constrict the plugged ampoules at 15 mm above the cotton plug using a constrictor. Alternatively, an air/gas glass blow torch can be used to manually constrict the ampoules.
7. Place the constricted ampoules in the secondary-drying apparatus, which consists of a vacuum system and a phosphorous pentoxide trap. Dry for at least 3 h, and seal each ampoule at the spot of constriction.
8. Test the sealed ampoules with a high-voltage spark tester to ensure the seal is intact by observing a purple to blue illumination inside the ampoule. Maintain the ampoules in the dark at room temperature. Some cultures require storage at 4 °C.
9. The shelf life of most bacteria ranges from 15 to 20 years. A shelf life of 30 years has been reported for members of actinomycetes and enterobacteria and *Staphylococcus* and *Streptococcus*. The method is described in use at the National Collection of Industrial Food and Marine Bacteria. However, few species, such as *Clostridium botulinum*, *Clostridium chauvoei (oedematiens)*, *Peptococcus*

heliotrinreciucans, *Spirillum serpens*, *Bacteroides melaninogenicus*, *Haemophilus canis*, *Leptotrichia buccalis*, *Mycobacterium microti*, and *Neisseria gonorrhoeae*, may have disappointing shelf lives.

For recovery, the stored ampoules are scored at the center of the cottonwood plug using a glass cutter and then heated in a Bunsen burner until red hot, and pressure is applied firmly to the score. The heat should crack the tube at the scored line. Aseptically open the ampoule, and remove the cotton wool plug. Then, add 2–4 drops of sterile distilled water or growth medium, replace the cotton plug, and leave for 30 min to rehydrate the suspension. Finally, inoculate a suitable growth medium with the suspension, and incubate under appropriate conditions. Check the growth and viability.

Lyophilization for Anaerobic Bacteria

A crucial problem for preserving anaerobic bacteria is maintaining viability. Based on the physiological characteristics of various anaerobic bacteria, preservation methods are divided into the following: (1) for obligate anaerobic bacteria, media are prepared and techniques must be used in an oxygen-free atmosphere (Hungate 1950), and (2) for anaerobic facultative bacteria, such as clinical isolates, the media are prepared and inoculated in air and may or may not require oxygen-free headspace (Rosenblatt et al. 1973). There are several strongly recommended media for growing anaerobic bacteria (Kirsop and Snell 1984), such as MM10, VL broth, and BP half broth. Cultures prepared for freeze-drying must be grown rapidly as Hungate cultures in a carbon dioxide headspace (Hungate 1950). It is recommended that for many bacteria, both MM10 and VL broth, supplemented with rumen fluid, fecal extract, liver extract, or lactate, are required. Hungate cultures in a suitable broth medium in screw-capped bottles at the growth stage are harvested by centrifugation (2500 rpm for 15 min). The supernatant is poured off, and 1 ml suspension fluid (7.5% glucose in horse serum sterilized by filtration) is immediately added. Again, the cultures in the screw-capped bottles are centrifuged (2500 rpm for 15 min), the supernatant is poured off, and 1 ml of sterile 16% glucose is added. The suspension should be made just before applying the primary freeze-drying step. The freeze-drying steps are the same as those for freeze-drying bacteria. All of the steps from the culture suspension preparation and freeze-drying process should be performed with minimum exposure to oxygen. The ampoules are stored in the dark at 2–10 °C. The viability varies and depends on the genus, species, and the degree of anaerobiosis required for the particular culture. In general, the viability of anaerobes preserved in this manner remains high for at least 10 years and even up to 22 years.

L-drying Method

L-drying method was first described by Annear (1958). This is a useful alternative method of vacuum drying to preserve bacteria that are sensitive to the freezing step of the common lyophilization method. The intrinsic feature of this process is that liquid cultures are prevented from directly freezing and drying. The method reported by Smith (Smith et al. 2001) is shown below:

1. Prepare the culture suspension by adding 2–4 ml of one of the three sterile suspensions of 100 ml horse serum and 33 ml Oxoid CM1 to an agar slant of the culture grown to the late log or early stationary phase, and mix. In case of medical bacteria, use a suspension of inositol serum (100 ml horse serum and 5 g of meso-inositol).
2. Aseptically add 0.1 ml of the cell suspension to each sterile ampoule using a sterile Pasteur pipette. Cut the sterile cotton wool plugs, and push down the ampoules using a sterile glass rod.
3. Connect the ampoules to the manifold, and clamp above a glass tank containing water at 20 °C, ensuring that the ampoules can be immersed in the water to a depth of 40–50 mm.
4. Connect the manifold through a diaphragm valve and phosphorous pentoxide trap to a rotary pump. Close the valve, and switch the pump on.
5. Open the valve very gradually and degas (i.e., bubble). To prevent violent bubbling in the ampoules, care must be taken to carefully control the rate of degassing by carefully manipulating the valve. When degassing is complete (approximately 5 min), open the valve fully. The contents of the ampoules will appear dry in additional degassing about 30 min.
6. Remove the ampoules from the manifold, and constrict the ampoules following the freeze-drying protocol for bacteria.

The shelf life of most bacteria preserved in this manner is approximately 10 years. Some bacteria such as *Spirilla* and *Azomonas insignis* that are sensitive to freeze-drying have been preserved successfully for 15 years using L-drying. Recovery is the same as for freeze-dried bacteria.

3.3.3.6 Algae

There are two common drying methods for preserving algae:

1. *Air-dried soil*: While not all stages of algal culture can be preserved by drying method, some can. Many algae tolerate adverse conditions in their natural environment, such as during zygotic or cystic stages. Using this method, zygotes of *Hydrodictyon africanum* have been successfully stored for 6 years, and cysts of *Haematococcus pluvialis* have remained viable after 27 years in air-dried soil.
2. *Lyophilization:* Freeze-drying techniques have been successfully employed for the preservation of algae (Holm-Hansen 1964). All of the steps are performed as in the standard protocol for bacteria. The cells are frozen in suspension liquid with an additive such as horse serum for *Bracteacoccus*, *Chlamydomonas*, *Chlorophyta*, *Cyanophyta*, and *Scenedesmus* and skim milk for *Chlorella*, *Phaeodactylum*, and *Stichococcus*. When compared with sub-culture and cryo-preservation methods, the freeze-drying method is not recommended because the average recovery rate is less than 5%. However, the successful lyophilization of the cyanobacterium *Nostocmuscorum* was reported with no observed reduction in viability during 5 years of storage (Holm-Hansen 1973).

Fig. 3.3 Liquid nitrogen tanks

3.3.4 Cryopreservation Methods

The preservation of living organisms by lowering their temperature reduces the metabolism rate until, when all internal water is frozen, no further biochemical reactions occur, and the metabolism is suspended (Franks, 1981). Temperatures of −20, −30, −40, −70, −140, and −196 °C have been used, but in general, temperatures above −30 °C give poor results. Refrigerated storage at −70 °C and storage in liquid nitrogen at −140 °C (nitrogen vapor phase) or −196 °C (nitrogen liquid phase) has been used for a variety of different microorganisms, including bacteria, fungi, yeast, actinomycetes, algae, mycorrhizae, and viruses (Fig. 3.3). Although little metabolic activity takes place at low temperatures, the recrystallization of ice can occur, resulting in damage to the cell structures during storage (Douzou 1982). Thus, adequate care must be taken during freezing and thawing, so the culture does not undergo phenotypic or genotypic changes. Adding cryoprotectants, such as glycerol, DMSO, skim milk, meso-inositol, and animal sera, to the culture suspension helps decrease damage. Cryopreservation is long term and does not require skill or complex techniques, but it does require expensive equipment and electronics, as well as a nitrogen supply. Cross-contamination can occasionally occur.

3.3.4.1 Filamentous Fungi

Storage on Agar Slopes at −20 °C

Agar slopes of fungi cultured at the mature or well-sporulated stage are maintained at −20 °C in deep freezers. For recovery, a small portion of the frozen colony is aseptically placed on a suitable agar medium at room temperature. The frozen stock culture is returned to the freezer as quickly as possible to avoid thawing. The shelf life of these cultures is 6–12 months. In some cases, such as *Aspergillus* and *Penicillium*, cultures can survive for 5 years.

Storage in Cryovials at −80 °C

Cultures of a wide range of fungi are stored successfully in deep freezers at −80 °C. It is a long-term preservation method when liquid nitrogen is not available. The shelf life varies from culture to culture over a large range (4–40 years). The disadvantages of this method are its dependency on electricity and expensive deep freezers and screw-capped cryogenic vials. Preparing the culture is simple. Spore or mycelial suspensions are made in 10% sterile glycerol or DMSO. An aliquot of 0.5 ml of the culture suspension is added aseptically to a sterile cryogenic vial and capped tightly. The vials are frozen at rate of 1 °C min^{-1} until they reach −35 °C (~35–40 min). Then, the vials are placed in a deep freezer at −80 °C. For recovery, each stored vial is used only once by thawing in a 37 °C water bath before inoculating on the suitable medium under appropriate conditions.

Storage in Liquid Nitrogen

Storage in liquid nitrogen (−139 °C in vapor and −196 °C in liquid form) is the most effective long-term preservation method. The shelf life of stored cultures is quite long in comparison with other methods. Under these conditions, the dormant cell does not undergo any phenotypic or genotypic changes. The method can be applied to both sporulating and non-sporulating cultures. At the Commonwealth Mycological Institute (CMI) culture collection, UK, 2900 of the 3286 cultures preserved in this way survived up to 14 years (Kirsop and Snell 1984). There are several storage containers other than glass ampoules that can be used successfully in liquid nitrogen vapor, such as plastic cryogenic vials with screw caps or plastic cryogenic straws. Care must be taken to avoid leaking containers that can result in cross-contamination. The disadvantages of this method include the high costs of nitrogen-controlled containers and a continuous supply of liquid nitrogen. If the supply of nitrogen fails (or the double-jacketed, vacuum-sealed storage vessels corrode and rupture), then the whole collection can be lost. The method applied at CABI (Smith et al. 2001) is as follows:

1. Aseptically prepare fungal suspensions in 10% sterile glycerol, and dispense 0.5 ml aliquots into cryovials (for non-sporulating fungi, plugs of mycelia can be cut and be placed in the cryotubes with 5 ml sterile glycerol). If the culture does not survive in glycerol, then 10% DMSO or a mixture of 5% DMSO and 8% glucose may be used (Smith 1983).

2. Seal cryovials with screw caps. For glass cryotubes, heat and seal using an air–gas torch before checking for leakage.
3. Keep the cultures at 5 °C for 1 h to allow the cells to equilibrate in the glycerol.
4. Cool the prepared cultures at a rate of −1 °C min^{-1} (common for many fungi) from 5 to −50 °C.
5. After the cultures are frozen (−50 °C), transfer them to the liquid nitrogen vessel.

For recovery, unscrew the cap of the cryovial or open the glass cryotube as in the freeze-drying technique before inoculating onto a suitable growth agar medium incubated under the appropriate conditions. Determine the viability and check for contamination.

3.3.4.2 Mushrooms

Freezing is applicable for storing a wide number of mushrooms. The method is preferred for non-sporulation mushrooms. In general, mushrooms can be stored at different freezing temperatures, such as −20 and −70 °C, and in liquid nitrogen at −139 °C (vapor phase) and −196 °C (liquid phase). When storing *Agaricus bisporus* (U-3) and *Pleurotus florida* (PAU-5) at −20 and −196 °C (in liquid nitrogen), for more than 6 months, the latter is the best storage temperature (Kaur et al. 2011). Although there were no differences in the shape, color, or other morphological characteristics of the fruit bodies formed by cultures stored at various temperatures, when the cultures were preserved in glycerol, in liquid nitrogen at −196 °C, in a deep freezer at −20 °C, or at room temperature, declines in endoglucanase enzyme-specific activity of 13.0, 7.6, and 80.2%, respectively, were observed after 20 days of incubation. Additionally, Suman and Jandaik (1991) reported no apparent changes in physiological or morphological characteristics of other mushrooms stored in liquid nitrogen for 10 years. The protocol for storing mushroom mycelia is the same as for filamentous fungi (Sect. 3.3.4.1) with a minor modification in preparing mushroom mycelial samples. The strains are sub-cultured onto complete agar medium plates and allowed to grow for ~10–14 days at 25–28 °C. After the mycelia have fully grown on the plates, they are cut into very small inocula using a hollow stainless-steel tube at 2–3 mm in diameter. The mycelial inoculum is aseptically placed in cryotubes containing 5 ml sterile 10% glycerol. If the culture does not survive in glycerol, then 10% DMSO or a mixture of 5% DMSO and 8% glucose may be used (Smith 1993).

3.3.4.3 Mycorrhizae

Cryopreservation is the most practical and reliable for the long-term storage of mycorrhizae.

- *ECM*: Several cryopreservation methods for storing ECM cultures have been developed recently. Cultures are stored by freezing in cryovials as per the cryovial

protocol (Homolka et al., 2003). The isolates are grown on medium supplemented with 5% glycerol (v/v) at 24 °C. Sterile plastic straws open at both ends are used to sample the agar colonized by the fungal hyphae. The straws are transferred into sterile cryovials, sealed, and frozen in a programmable freezer to −70 °C, with a controlled slow cooling rate of −1 °C min^{-1}. They are then plunged into liquid nitrogen. After storage, the cryovials are thawed rapidly at 37 °C. Using this protocol, *E. clandestinum* presented a 100% survival rate, while *S. citrinum* and *S. verrucosum* isolates presented 50% survival rates. These fungi maintained their extracellular laccase production. Freezing the cultures on filter paper discs has been applied successfully to 13 ECM isolates (Stielow et al. 2011). The protocol involves growing the fungal isolates on charcoal filter paper strips (CFSs) placed on the surface of a culture medium for 3–5 weeks. The CFSs are collected in a sterile petri plate, incubated in 10% sterile glycerol (v/v) for 1–2 min and transferred into cryovials by layering the CFSs on top of each other. The closed cryovials were placed for 24 h in the gas phase of a liquid nitrogen tank (i.e., at a cooling rate of approximately −1 to −10 °C/min until reaching between −120 °C and −140 °C), before being directly transferred into liquid nitrogen. The cryovial protocol below (Crahay et al. 2013) efficiently stored 100 ECM fungal isolates and is similar to the method applied to mushrooms in Sect. 3.3.4.2 (Suman and Jandaik 1991):

1. ECM fungal isolates are grown on agar medium in petri plates and incubated under appropriate conditions for 2–4 weeks.
2. A mycelial plug of ~4 mm in diameter is taken from the margin of the growing colony and inoculated into a 2 ml sterile polypropylene cryovial containing 750 µl of sterilized (121 °C for 15 min) suitable agar medium poured at a slope.
3. Cryovials are incubated at 22–23 °C in for 7–9 weeks.
4. At 1–2 h before cryopreservation, 500 µl sterilized (121 °C for 15 min) glycerol cryoprotectant solution (10% v/v) is added to the cryovial.
5. The cultures are cryopreserved following a controlled decrease in temperature (−1 °C min^{-1} from 4 to −50 °C).
6. The cultures are directly transferred into a freezer at −130 °C.
7. For recovery, the ECM fungal isolates are directly thawed in a water bath at 35 °C for 2 min. The culture plugs are transferred to the centers of petri plates containing 30 ml suitable medium and incubated at the appropriate temperature.

Some parameters appear paramount for the long-term preservation of ECM fungi:

1. The preconditioning of the culture prior to preservation: The fungi are cultured on a carrier or in a cryovial to avoid excessive manipulations may damage the hyphae before preservation. Flooding the culture with a cryoprotectant (1–2 h before cryopreservation) can increase the survival rate of the fungi (Lloyd 1994).

2. The age of the culture: Cultures in the late phase or stationary phase of growth are most often better adapted to survive freezing or freeze-drying than young cultures (Lloyd 1994).
3. The cryoprotectant used: Glycerol appears to be the most reliable (and is the most used) cryoprotectant for ECM fungi, as well as for the majority of filamentous fungi (Cavalier-Smith 1998).
4. The cooling rate: The slow cooling of the organism (i.e., ~−1°C min^{-1}) appears to be appropriate for the majority of ECM fungi. Mycelia, which are the predominant form of ECM fungal colonies on synthetic medium, are rich in water. Thus, it is essential to dehydrate, at least partially, the hyphae to avoid injury caused by water crystallization.
5. The thawing rate: Fast thawing by direct immersion in a water bath (i.e., at a temperature between 35 °C and 38 °C) is often reported to prevent the risk of ice recrystallization occurring during a slow thaw.

- *AMFs*: For in vivo cultivation, soils containing isolates of *Glomus*, *Acaulospora*, and *Gigaspora* were first dried at room temperature and then directly stored at −80 °C (Kuszala et al. 2001). The cultures survived for several months. In another case, Kuszala et al. (2001) succeeded in cryopreserving 15 isolates belonging to *Glomus* and *Acaulospora* by immersion in liquid nitrogen.

For in vitro cultivation, Addy et al. (1998) was the first to demonstrate that the hyphae of *Glomus intraradices* were able to survive at temperatures below −12 °C when slowly cooled before freezing (Lalaymia et al. 2014). A method of cryopreservation was then developed (Declerck and Angelo-Van Coppenolle 2000). It was based on the encapsulation of spores in alginate beads, followed by incubation in trehalose (0.5 M) before freezing at −100 °C using a two-step temperature decrease of −1 °C min^{-1} from 20 to −35 °C and −18 °C min^{-1} from −35 to −100 °C. The advantage of in vitro preservation of AMF cultures is the production of pure contamination-free inocula. However, in vitro cultures are only successful for the limited number of species.

The method for the cryopreservation of AMFs developed by Lalaymia and co-workers (2014) should be applicable for both in vivo and in vitro AMF. It is as follows:

1. *Preparing the culture*

   For in vitro: Gelling medium, containing spores and roots of a 5-month-old in vitro culture, is extracted from the petri plates and poured into 100 ml of sterilized (121 °C for 15 min) deionized water and subsequently blended two times for 30 s at the 20,000 rpm in a sterilized (121 °C for 15 min) mixer.

   For in vivo: Pot-cultures that are at least 5-month-old are sampled. Spores are collected by wet sieving and decanting, while roots are collected with forceps and blended in a mixer in 100 ml deionized water for 30 s at 20,000 rpm and then filtered as above. The spores and the supernatant of the blended roots are mixed together.
2. The mixture is filtered using a sterilized (121 °C for 15 min) nylon mesh (40 mm).

3. The supernatant (i.e., spores and mycorrhizal/non-mycorrhizal root pieces) is encapsulated in a 2% (w/v) solution of sodium alginate (50 ± 5 propagules in each bead).
4. The encapsulated propagules are incubated in trehalose (0.5 M) overnight.
5. They are then dried at 27 °C for 48 h (until the bead water content is approximately 8.1 ± 4.6%).
6. The beads are transferred in 2 ml cryovials.
7. The cryovials are cryopreserved in a freezer at −130 °C following a two-step decrease in temperature: a fast decrease (~−12 °C min^{-1}) from room temperature (20 °C) to −110 °C, followed by a slow decrease in temperature (~ −1 °C min^{-1}) from −110 to −130 °C.
8. *Recovery:* Thaw the encapsulated AMF propagules by directly plunging in a water bath at 35 °C.

In vitro The beads are dropped in sterilized (121 °C for 15 min) MSR medium, cooled in a water bath to 40 °C, and then incubated at 27 °C for germination. After 4 weeks, beads containing germinated propagules are associated with a transformed root under in vitro culture conditions to reinitiate the fungal lifecycle.

In vivo The encapsulated propagules are placed directly in contact with plants in pots containing a sterilized (2 × 15 min at 121 °C, with a 12-h interval) substrate. The plants are grown for at least 8 weeks in a growth chamber before the fungal viability is assessed.

From the above cryopreservation protocols, the following six important storage factors were discerned:

1. The culture age is independent of the production mode (i.e., in vitro or in vivo). AMF propagules isolated from cultures in the stationary growth phase are the most suitable for the long-term preservation. At the late growth phase (frequently associated with stress conditions), the fungus accumulates cellular compounds, such as trehalose (Van Laere 1989), polysaccharide, and glycoproteins, to protect the intra- and extracellular mycelial integrity.
2. Drying may reduce ice crystallization during freezing. The natural cryoprotectants produced during drying may reduce ice crystal size or convert the fungal cytoplasm into a glassy state during rapid freezing, thus improving the chances of survival (Tan et al. 2007).
3. *The carrier*: AMF propagules cultured in vivo are usually preserved in the soil substrate in which they are produced. Encapsulation in alginate beads was reported as mandatory for the cryopreservation of in vitro-cultured AMFs (Declerck and Angelo-Van Coppenolle 2000).
4. *The cryoprotectant*: For in vitro-cultured AMF propagules, the use of trehalose (0.5 M) as a cryoprotectant is recommended, while the in vivo-cultured propagules could be preserved in dried soil without a cryoprotectant. Trehalose has the capacity to interact with phospholipids of the cell membrane to maintain their fluidity during freezing and desiccation (Crowe et al. 2001).

5. *Cooling rate*: Fast cooling seems to be preferred by AMFs (Kuszala et al. 2001). Identically, Lalaymia et al. (2014) reported that the most effective preservation of in vitro-produced AMF isolates occurred by direct cooling in the freezer. This may result from AMF propagules (i.e., spores and/or vesicles) being less rich in water as compared with hyphae.
6. *Thawing of AMFs*: Fast thawing by placing cryopreserved AMF propagules cultured either in vitro or in vivo in a water bath at 35 °C is generally preferred to prevent the risk of ice recrystallization after cryopreservation.

3.3.4.4 Yeast

Freezing Cryovials/Ampoules in Liquid Nitrogen
This is a two-stage freezing method in which the cells are initially cooled for dehydrating at −30 °C and then cooled at an uncontrolled rate to −139 °C (vapor form) or −196 °C (immersed in liquid nitrogen). The method has been used for a fairly wide range of yeast, including *Bulera*, *Candida*, *Hansenula*, *Lipomyces*, *Kluyveromyces*, *Pichia*, *Rhodosporidium*, *Rhodotorula*, *Saccharomyces*, *Saccharomycodes*, *Schizosaccharomyces*, and *Zygosaccharomyces*. The shelf life of stored yeasts is expected to qualify as long-term preservation. The National Collection of Yeast Cultures detected no decreases in viability levels in test strains over a period of 26 months, and others recorded good survival rates for up to 4 years. The average survival levels are 65% for *Saccharomyces*, 73% for *Candida*, and 74% for *Brettanomyces*. The method is described as follows:

1. Aseptically prepare the yeast suspension in sterile glycerol: The yeast culture is grown on YM broth (Difco, 0711-01) for 72 h. Prepare each 1 ml sample of the yeast suspension in sterile cryovials/ampoules by mixing 0.5 ml of yeast culture containing 10^6–10^7 cells ml^{-1} with 0.5 ml of filter-sterilized 10% glycerol (0.45 μm pore size). The final concentration of glycerol is ~5%. Care should be taken when screwing the caps to prevent the leakage of liquid nitrogen into the samples.
2. Primary freezing: The cryovials/ampoules are frozen to −30 °C by placing them in a deep freezer. The cooling rate is not critical in this method. Keeping them at −30 °C for 2 h allows the cells to dehydrate.
3. Secondary freezing: All samples are transferred to, and immersed in, liquid nitrogen containers. Care is taken to prevent the samples from thawing.

The recovery protocol is the same as for fungi.

Cryopreservation Straw Method This is an alternative method of freezing yeast cultures. It is used by many culture collections and is recommended by the WFCC.

1. Culture growth: 10 ml YM medium (Difco broth, 700101) for 72 h at 25 °C on a shaker.
2. Equal amounts of the inoculum and 10% sterile glycerol are mixed in a sterile bottle.

3. Remove prepared sterile straws (4 mm × 2.5 cm, sealed at 10 mm from one end; straws are placed in a glass petri dish and sterilized by autoclaving at 121 °C for 15 min.)
4. Add the culture suspension to the straw to approximately two-thirds of its capacity (within 3 mm of the open end).
5. Seal the open end.
6. Test the straw for leaks by holding the sealed straws with forceps and gently squeezing.
7. Place six straws in each 1.8 ml cryotube (mark the cryotubes or straws with the freezing date).
8. Primary freezing: Place the filled cryotube in a methanol bath that has been precooled to −30 °C.
9. Secondary freezing: Transfer the pre-cooled cryotubes to a liquid nitrogen container, and place in a rack. Remember to remove the excess methanol from outside of the cryotubes to prevent it from freezing the tubes to the rack while immersed in the liquid nitrogen.

   For recovery: Remove a single straw, and transfer to a 35 °C water bath. Remove from the water bath, grip one end, and sterilize in 95% alcohol. Suspend the cells by squeezing the straw several times. Cut off the sterilized end with sterile scissors, and transfer the culture suspension to an appropriate growth medium.

3.3.4.5 Bacteria and Actinomycetes

Cryopreservation Using Glass Beads

Storing bacteria at −60 to −86 °C in glass beads is a simple and inexpensive method that avoids the damage that can occur to the cell that is subjected to repeated freezing and thawing. The technique allows individual beads to be removed without thawing the entire tube. The resulting shelf lives are reported to be 7 years (Feltham and Sneath 1982).

1. Prepare the sterile vials containing washed beads. The screw-capped glass 2 ml vials (Creative Beadcraft Ltd.) containing 20–30 prepared 2 mm glass beads (R.W. Jennings Ltd.) are sterilized by autoclaving at 121 °C for 15 min. Slightly loosen the caps prior to, and screw down right after, autoclaving. Prepare the beads. First, the beads are washed in tap water with detergent, followed by dilute 0.1 M HCL to neutralize alkalinity. Then, they are washed again with tap water until reaching the tap water's pH. A final wash in distilled water is performed before the beads are dried at 50 °C in an oven.
2. Growing the bacteria. Bacteria should be grown on the most appropriate medium under optimum conditions. A solid medium plate is recommended to prevent the risk of contamination.
3. Prepare a culture suspension with a cryoprotectant. Aseptically withdraw 1 ml of sterile suspension medium [10 ml bottles of with 15% nutrient broth (Difco

Laboratories) are autoclaved at 121 °C for 15 min], and add to the culture plate. A wire loop is used to emulsify the cell suspension.
4. Distribute the culture suspension to glass bead-containing vials. The culture suspension is aseptically dispensed to glass bead-containing vials using a sterile Pasteur pipette. After the beads are thoroughly moistened, the excess suspension should be removed from the vial. Excess suspension in the vial makes it difficult to remove individual beads after storage.
5. Freezing glass bead vials. The vials are placed in suitably sized trays that are transferred to a commercial deep freezer at 60–80 °C (−70 °C is recommended).

For recovery: A vial is removed from the freezer, and one bead is then removed using a mini-spatula sterilized by flaming in alcohol and then cooling. The bead is rubbed over the surface of a suitable solid plate medium and incubated under the appropriate conditions. Determine the viability, and check for contamination.

Cryopreservation by Freezing Cryovials of Culture in Liquid Nitrogen
This method is an alternative for cultures that cannot survive lyophilization or the L-drying method and for patent deposits, sensitive mutants, genetically manipulated strains, and all bacteriophages, including other cultures with phage particles. It is recommended to add cryoprotective agents using the following criteria: (1) nontoxic to the cell, (2) penetrates the cell membrane easily, and (3) binds either an electrolyte that increases in concentration during freezing or water molecules to delay freezing. Common cryoprotectants include glycerol and DMSO. The shelf lives for a wide range of bacteria are over 10 years. The basic steps are simple.

1. Cells are grown on a suitable broth medium to mid or late phase.
2. The cell count is adjusted to 2–6 $\times$ 10^6 cells/ml. Sterilized glycerol or DMSO is added and mixed to a final 5% concentration.
3. The cell suspension is aseptically dispensed in 0.5 ml aliquots into 2 ml sterile cryovials or cryotubes. These are maintained at 30 °C for 30 min to allow for cell dehydration. The samples are checked for leaks.
4. Primary freezing: The temperature is cooled to −30 °C at a rate of 1–3 °C/min.
5. Secondary freezing: The samples are transferred to a liquid nitrogen container. It is recommended to use the vapor phase.

For recovery: The cryovials (or cryotubes) are removed and immediately placed in a 37 °C water bath to thaw the cultures as rapidly as possible. A sterile wire loop is used to streak the culture on a suitable agar plate medium under the appropriate conditions.

3.3.4.6 Algae

Compared with other groups of microorganisms, relatively little research has been carried out on the development of long-term preservation methods for algae and cyanobacteria. Cryopreservation has been successfully employed to maintain algae (Morris 1981). Most protocols use a simple two-step system with controlled/semi-

controlled cooling from room temperature to a subzero holding temperature before immersion in liquid nitrogen. There are several different cryoprotectants in use, but DMSO and methanol have been reported to protect many algae better than glycerol (Morris 1981). Care should be taken to remove DMSO when recovering aliquots, because even trace amounts can inhibit photosynthesis. However, the majority of protocols in current use have been developed with a pre-freezing culture regime, cryoprotectant choice (DMSO or methanol), cooling rate, and thawing regime to minimize the damage to algal cells and maximize the viability level.

The following method for algal preservation is in use at the Culture Collection of Algae and Protozoa, UK:

1. Grow the culture in the appropriate medium under controlled conditions. The 50 ml flasks, containing 30 ml medium, should be incubated at 15 °C under suitable light (dark) conditions. Generally, 30 days are a standard interval for a culture to reach the late log or early stationary phase.
2. Aseptically transfer 15 ml of culture to sterile centrifuge tubes, and harvest the algae by centrifugation at 500 rpm for 10 min at an ambient temperature. Collect the algae, and resuspend in 15 ml fresh sterile medium.
3. Add 10 ml of sterile 10% DMSO to 10 ml of dense algae, and gently mix to a final 5% DMSO culture suspension.
4. Aseptically dispense 1 ml of the culture into sterile 2 ml cryovials/cryotubes. Screw caps down, or seal the cryovials, and maintain at room temperature for 5 min.
5. Primary freezing occurs by transferring the culture vials to a pre-cooled freezer (−30 °C) and incubating for 15 min.
6. Secondary freezing occurs by transferring the culture to a liquid nitrogen container and immersing it in liquid nitrogen.

For recovery: Thaw the vial by removing and placing it in a water bath at 37 °C. Wipe the vial with 70% (v/v) ethanol to sterilize the outside, transfer the whole content of the culture to a bottle containing 30 ml suitable sterile medium, and incubate under appropriate conditions.

3.3.4.7 Preservation Methods for Microbial Communities

In the environment, microorganisms are extremely important to the recycling of elements and nutrients, to balancing trophic chains, and to vital physiological activities in plants and animals, as well as to the conservation of natural habitats. It is crucial to store cultures in mBRCs, but they do not conserve the full diversity of microorganisms found in nature. Functional diversity is very important in the ecological assessment of microorganisms within the ecosystem, mainly because little is known about the relationship between the structural and functional diversity levels of these microorganisms (Yamanaka et al. 2003). Because of advances in technology in metagenomics, metaproteomics, and metatranscriptomics as well as molecular biology, including bioinformatics, much progress has been made in

understanding and applying microbial exosystems, such as methanotrophic co-cultures, oxygen-limited autotrophic nitrification/denitrification biofilms and anaerobic AOBs, human intestinal microbial ecosystems, and rumen microbial ecosystem, from various microbial communities (microbiomes) in environmental, industrial, and healthcare-related fields. The microbiomes are likely mixed cultures of both uncultivable and cultivable isolates with many differences in physiological and biochemical characteristics. The assessment of stored microbiomes is not the same as for pure isolates in mBRCs. A metagenomic analysis is the most common method, compared with metatranscriptomic and metaproteomic analyses for assessing the quality of stored microbiomes. The same stored microbiomes may not give the same results when different analysis methods are used. In recent decades, developing methods for storing microbiomes has received more attention.

Yu et al. (2015) stored switchgrass-degrading microbial communities for 56 days at −80 °C with glycerol and DMSO as the cryoprotectants. The relative abundances of *Firmicutes* and *Actinobacteria* were also greater after being stored with DMSO. The storage conditions were not favorable for *Anaerolinea*, *Roseiflexales*, and *Steroidobacter* but did favor *Micromonosporaceae*, *Chelatococcus*, and *Thermobacillus.* Samples preserved with DMSO and glycerol did experience a consistent shift in community composition, although dominant microorganisms were retained in the active communities. In another study, microbial communities in marine sponges were stored by both lyophilization and freezing with or without RNAlater solution. Total DNA and RNA from stored samples were extracted by various methods for a denaturing gradient gel electrophoresis analysis of the community's 16s rDNA genes. It is likely that lyophilization and freezing in liquid nitrogen provide better results (Simister et al. 2011). When preserving microbiomes found in infant fecal samples for sequencing 16s rDNA and oligosaccharides, Lewis et al. (2016) reported that lyophilization was an acceptable method of sample preservation for the purposes of studying microbial communities. The optimization of the cryopreservation of three microbiomes, methanotrophic co-culture, oxygen-limited autotrophic nitrification/denitrification, and human fecal material, was studied based on analyzing operational taxonomy units before and after cryopreservation (Kerckhof et al. 2014). The cryopreservation protocols that used a cryoprotectant (DMSO, trehalose, or tryptic soy broth) succeeded in preserving both community structure and functionality. In addition, Gray and co-workers attempted to compare DNA preservation methods for mixed environmental bacterial samples using DNA, RNAlater, DMSO-EDTA-salt, FTA card, and FTA Elute cards (Gray et al. 2013). The liquid-based preservatives (DNAgard, RNAlater, and DMSO-EDTA-salt) outperformed the card-based methods. Generally, long-term preservation methods, such as lyophilization and cryopreservation in liquid nitrogen or freezing (−80 °C), are preferred for microbiomes. Glycerol, DMSO, or trehalose should be used as a cryoprotectant.

3.3.5 Providing Qualified Microbes as Starting Genetic Resources for Scientific, Agricultural, Environmental, Medical, and Pharmaceutical Research and Applications

3.3.5.1 Culture Quality

An important service of mBRCs in supporting life science and biotechnology development is distributing preserved cultures to users. The user expects the culture to have good qualities: (1) reference cultures come from a reliable source, (2) standardized cultures produced from expertly preserved authenticated seed stocks with appropriate traceability, (3) cultures are as described with valuable information documented, and (4) an "authentic" strain. Before being supplied, the culture should undergo the following chain of events: (1) received from depositor; (2) viability, purity, identification, and identity (authenticity) confirmed; and (3) preserved as a stock until supplied. The quality of a culture depends on continual working practices, equipment, media, reagents, personal skills, and experiences from deposition to distribution. Thus, all of the mBRCs need to address key functions: authentication, best practices in preservation and supply, and confirming the validity of the associated information provided (Smith et al. 2014). The best industrial practices based on ISO-9001:2000 are recommended. For quality assurance, confirming the identity of the culture (the culture being distributed should be the same as deposited to mBRCs) is not easy. This is very important for the reproducible performance of the culture. The identity needs to be confirmed before and after preservation, as well as during storage, by experienced experts and taxonomists. In some cases, the depositor is required, if available. Supporting techniques, such as sequencing, fingerprinting, and analyzing protein profiles by MALDI-TOF, are applicable and useful. To reduce the variation in a stored culture, it is advisable to minimize the numbers of transfers or generations from the original culture, where this is appropriate. The mBRC must use master (or seed) and distribution stocks. The mBRC must produce the master stock from the original culture. This master stock must be used to generate the distribution stock. The mBRC must use the distribution stock for distributions to users.

3.3.5.2 Supplies

User Requirements

In general, a user (individual or organization) is requested to fill and sign the Order and Accession forms from the mBRCs. The form contents should have some clear main points: (1) user's official contact information (contact name, delivery address, and telephone/fax number); (2) the form of culture (living or freeze-dried ampoules); (3) written and signed documentation proving that the user has appropriate containment facilities and authorization to import and handle such a culture, in particular, safety and security requirements needed for handling plant, human, and animal

pathogens; and (4) in compliance with local, national, and international legislation, such as the CBD and Nagoya Protocol on access and benefit sharing (ABS) for bioresources. In some cases, an MTA is added to the forms.

When ordering, users are advised not to quote accession numbers unless they are sure that a particular culture is most suitable for their purpose. It is frequently more satisfactory to state the purpose for which the culture is required and to leave the choice of strain to the collection staff.

Available Cultures

It is understood by users that mBRCs should have the capacity to provide the timely and reliable delivery of catalogued strains. However, the number of catalogued strains and the total number of holdings are not the same. The available cultures are documented and available for distribution. For many stored cultures, the long-term preservation by lyophilization or cryopreservation is time-consuming and labor-intensive in checking viability, purity, and identity. If the situation changes, the user should be informed.

Pricing

The monetary value of the cultures needs to be set. The price depends on the level of difficulty in handling the culture, its preservation method, characterizing properties, and confirming its identity and associated information. In some cases, the cost is as high as €3000 (Smith et al. 2014). A discount can be applied for teaching and scientific activities and bulk purchases. Normally, a value-added tax and transportation fees are not included in the price. Gratis exchange agreements are in use in many mBRCs. However, the memorandum of understanding is applicable for related organizations and individual users.

Restrictions

All culture parcels are required to be opened in an appropriate laboratory.

If the strain is a nonindigenous pathogen, the customer has to obtain the relevant license before the strain can be delivered. For cultures belonging to hazard groups (2 or 3), the appropriate containment facilities are needed for safety and security. The user should be informed of any special restrictions and the relevant paperwork that is required from them, prior to providing the requested pathogen cultures.

Delivery and Packing

The mBRC must provide at least the following information to the user: (1) biological material identifier, accession number, and batch number; (2) an estimate of the shelf life, storage conditions, storage instructions and, if appropriate, growth conditions; (3) instructions for opening ampoules or vials (when appropriate and in all cases where materials are being provided to new users); (4) a safety data sheet, including the containment level required for handling the biological material, disposal measures, and measures to take in case of spillage; and (5) an MTA, an essential requirement to protect the IPR and mandatory where they are required. They are used to relay the depositor's and/or country of origin's requirements on the use of the culture. The packing of biological materials and its transport by postal and other transport services is controlled by international and regional agreements and national

laws. For noninfectious microorganisms of Risk Group 1, the cultures can be sent by airmail and other means of transport. For infectious microorganism, mBRCs should follow the WHO Guideline on International Regulation for the Packing and Transport of Infectious Substances. However, pathogenic microorganisms that qualify as dangerous goods cannot be sent by air in UN-certified packages if the postal services of the countries through which it passes do not allow the microorganisms in the postal system. Currently, there are few private carriers that transport dangerous goods internationally, with the additional costs of packing and documentation needed at the airport. The carrier is responsible for the shipment, correct packing, documentation, marking, and labeling (Smith et al. 2001).

3.3.5.3 Repositories of Microbial Genetic Resources and Receiving Safe Deposits for Publication and the Protection of Intellectual Property Rights

Servicing deposits of cultures from depositors (individual and organizer) is a useful activity of mBRCs. There is no culture collection that has enough diverse microbes to meet the increasing demand for life science-related activities. Deposited cultures are a source of enrichment for mBRCs. For the depositor, before publishing and applying for the patent, the culture is required to be deposited at an mBRC. In addition, there are other advantages to the depositor: (1) safe preservation, (2) worldwide accession, (3) removal of the burden of distribution, and (4) exchanging cultures. For safe deposits, to ensure long-term viability, cultures need to be stored in freeze-dried ampoules or cryopreserved in liquid nitrogen. The depositor is required to fill the deposit form. The culture to be deposited should be provided with the following information: (1) name, number, or other identifier; (2) depositor's name and address; (3) original source from which the culture was isolated, as well as the place and date of isolation; (4) growth media and conditions and preservation conditions; (5) hazard information and biosafety level; (6) conditions and any restrictions on the distribution of the deposited culture; and (7) the rights of the depositor and mBRC (in compliance with international and national regulations). The price and termination conditions need to be set, including maintenance services and interval quality checks. Not all deposits receive an accession number and are maintained. Sometimes viability and identification checks are not successful. The failure rate is more than 10% annually in some mBRCs. The problems could relate to contamination, identification, or reculture.

3.3.5.4 Conducting R&D on Microbes

Based on the techniques, skills, and knowledge required for checking the viability, purity, and identities of cultures, mBRCs should be more involved in conducting R&D activities on stored microorganisms. The output from these activities would

make the cultures more valuable by adding information and increase the value of the mBRC as a microbiology research unit.

Enrichment of Biodiversity and Isolation Techniques

To meet the needs of different users, mBRCs need a large number of diverse cultures, especially reference cultures, such as type cultures. The collection should be independent from internally and externally deposited and exchanged cultures. The curators should be aware of the requirements for cultures from customers, especially national users. Strains of cultures from indigenous sources are crucial for enriching the size and diversity of the collection. Discovering novel cultures is a good enrichment activity. The same sample taken for isolation, testing using different techniques, and growing in media may give rise to various kinds of cultures and increase the biodiversity level. In our laboratory, novel species of filamentous fungi are isolated by directly selecting single spores with a Skerman's micromanipulator. Novel isolation techniques should be developed at the mBRCs.

Taxonomy and Identification

Isolates require a taxonomic analysis and identification to determine whether the isolate represents new taxa or known one. For new taxa, the isolate may have novel properties and applications. For known taxa, the available knowledge and experiences relating to the taxa should be useful to direct further studies on the isolate. Performing taxonomic analyses and identification experiments requires manpower, expertise, and expensive equipment. In fact, not all culture collections (especially private and in-house collections) can implement these activities. However, the trend of many public mBRCs, such as the Thailand Bioresource Research Center, Thailand; VTCC, Vietnam; and Leibniz-Institute DSMZ–German Collection of Microorganisms and Cell Cultures, Germany, includes performing R&D. In total, 330 culture collections provide identification services (Smith et al. 2014). Based on progress in supporting fields, such as molecular biology and bioinformatics, research activities are not limited to individual pure cultures but have expanded to include the study of the biodiversity of microbial communities. The mBRCs need to update any changes in the nomenclature of the preserved cultures. The Leibniz-Institute DSMZ–German Collection of Microorganisms and Cell Cultures website (www.dsm.de) has a section "nomenclature up to date" that can be visited.

Characterization of Microorganisms

Many mBRCs have large collections of microorganism that include cultures that have not been characterized yet. While mBRCs cannot conduct intensive studies on all of the preserved cultures, some important information on the microorganisms that support its practical application are useful to users.

Microorganisms are useful in various fields of the life sciences. Their uses include (1) type strains (for bacteria) and ex-type strains (for fungi) that are used as reference strains for taxonomic studies, (2) enzyme producers, (3) secondary metabolite producers (vitamins and bioactive compounds, such as bacteriocins, fungicides, and antiviruses), (4) antibiotic producers (penicillin, tetracycline, and vancomycin), (5) food and fermented food production, (6) biocontrol agents (such as *Bacillus*

thuringinesis and *Bauveria bassiana*), (7) environmental microbes, (8) biorefinery and bioremediation, and (9) pathogens. To identify such properties, many screening methods are conducted, such as (1) screening enzymes, (2) screening antibiotics, (3) screening bioactive compounds, and (4) fermentation studies. The data obtained is added to each culture's profile and stored in a database.

3.3.5.5 Documentation and Databases

For public services, mBRCs need to make the preserved cultures and associated information accessible. Documentation and database are needed for all culture collections. All of the data needs to be maintained for all strains in written or electronic records. Depositors are responsible for assuring the quality of the data associated with deposited cultures. The data will be updated and maintained with the culture. The mBRCs can store data and produce catalogues (written or electronic form) based on authenticated and validated information. Data should also be retained for traceability in compliance with relevant national laws, regulations, and policies. For security, mBRCs should adhere to national data protection regulations. If mBRC data are made available online or on disc, nonconfidential information should be presented. The data for searching and retrieving information from the catalogue and the database must be identical. It is strongly recommended to use standard terminology and formats for data exchanges and protocols for data transmission. For more practical use of the data, a standardized uniform format should be used. Recently, WDCM minimum dataset (MDS) and recommended dataset (RDS) were developed by the Global Catalogue of Microorganisms project (Wu et al. 2017).

Many culture collections use common software programs (Excel or Access), but some are using software specific for mBRCs, such as iCollect software (TBRC: Thailand Bioresources Center) and BioloMICS software (CBS).

3.3.5.6 Performing Related Services and Consulting

Training

To efficiently operate and manage an mBRC, extensive knowledge, experience, and expertise, such as in general microbiology, taxonomy, molecular biology, and bioinformatics, are required. In addition, much progress in these fields has been made, resulting in conventional methods, techniques, and knowledge becoming outdated and replaced. Organizing training courses conducted by experienced specialists will provide the existing staff with new knowledge and techniques and also provide manpower for the sustainable development of the mBRC. However, conducting qualified technical training course is costly in manpower and facilities. Only ~280 of 700 WFCC members can offer training services (Smith et al. 2014). International training courses conducted by scientists from reputed mBRCs are practical. Conducting training courses for local young scientists improves the mBRC's reputation and can be supported by the government.

Consultancy

Technical Advice and Consultancy

It is notable that many users are not microbiologists. Thus, they may have some difficulties in handling and performing a study using microorganisms. The support from experienced staff should improve their experience. The contents of training courses vary. Many mBRCs conduct courses on some common topics with the available staff and facilities, such as (1) collection management, (2) isolation of microorganism, (3) preservation methods, (4) taxonomy and identification, (5) food safety, and (6) special techniques (Smith et al. 2001).

Consultancy on Related Policies and Regulations

Because of the fast progress of global science and technology, fostering internal and external collaborations is important for any country to exchange and update knowledge. A number of internal and external collaborations are established based on microorganisms. Policies and regulations for collaboration based on plant and animal use are more common than on microorganisms. In fact, policies and regulations should protect the sovereign and intellectual property rights, benefit from local biodiversity, and support internal and external collaborations in compliance with international and national laws and regulations under the light of the CBD and ABS. However, not all regulations that are applicable to animals and plants are suitable for microorganisms. Many plants and animals have a long history of traditional knowledge, while a good number of the microorganisms annually deposited represent novel species. It was reported that ~900 new fungal species and ~700 new bacterial species were described in a year (Blackwell 2011); however, they do not all have a ready application. Before use, those microorganisms may require much study before they have an effective use. This process may be risky, and it is not easy to evaluate the benefits of microorganisms. This issue should be addressed by policymakers for practical guidance.

3.4 International Collaboration in Asian mBRCs

3.4.1 International Collaboration, Access and Benefit Sharing Under the CBD and Nagoya Protocols on ABS

3.4.1.1 International Collaboration

In the long history of culture collection development, there is no culture collection that has a large enough holding and expertise to satisfy the wide range of user demands. The collaboration among mBRCs in the exchange of knowledge, expertise, experiences, and cultures is important for culture collection management. The mBRC collaborations have been established for the following different geographical areas: (1) national level, Thailand (Thailand Network on Culture Collection) and the Philippines (the Philippine Network of Microbial Culture Collection); (2) regional level, Europe (European Culture Collection Organization), Asia (the Asian

Consortium for the Conservation and Sustainable Use of Microbial Resources), and Latin American (the Latin American Association for Microbiology); and (3) global level, WFCC, Microbial Resource Centers, and WDCM. The worldwide development of organizations and the management of culture collections are strong indicators of the increasing demand for microbial resources in the life sciences. The WDCM website on 31 December 2016 listed 713 culture collections from 74 countries or regions holding 1,051,947 bacteria, 788,397 fungi, and a total of more than 2.5 million microorganisms. Among the culture collections, 232 from Europe, 250 from Asia, 181 from the Americas, 41 from Oceania, and 11 from Africa are registered with WDCM (Sharma et al. 2017b). Thus, the roles of international organizations are crucial. The WFCC Executive Board has recently updated the WFCC Guidelines for the Establishment and Operation of Collections of Cultures of Microorganisms. The dissemination of the WFCC guidelines at the local level reinforces awareness (http://www.wfcc.info/). In recognition of genetic resources for world development, the Organization for Economic Cooperation and Development in collaboration with the WFCC issued several guidelines for the management of mBRCs, including *guidance for the operation of Biological Resource Centers (BRCs)-2004*, *OECD best practice guidance for BRCs-2007*, and *certification and quality criteria for Biological Resource Centers* (www.oecd.org). To ensure the quality of provided cultures, many mBRCs are certified and accredited by ISO standards ISO9001:2000, ISO 9001-2001, and ISO 9001-2008 (Smith et al. 2014).

3.4.1.2 ABS Under the CBD and Nagoya Protocol

Because culture collection collaborations involve living cultures, they differ from other collaborations. A living culture is not limited in commercial research activities, and it may be the starting genetic source for developing a commercial product or applying for patent. The Budapest Treaty on the international recognition of the deposit of microorganisms for the purpose of patent procedures was the first international attempt to support culture exchanges and to protect the intellectual property rights of the depositor.

The CBD in 1993 was a milestone marking the worldwide recognition of protecting the Earth through the conservation of biodiversity for bio-economic development. The main content of the convention is found in the following three objectives: (1) conservation of biodiversity, (2) sustainable use of its components, and (3) fair and equitable sharing of benefits arising from the utilization of genetic resources. Having an agreement between the user and provider on benefit sharing is crucial for collaborations. The CBD was a great achievement for international legislation on collaborations based on genetic resources. It provided an open platform for collaboration with key concepts and guidelines in Articles 15, 16, and 17. However, the procedure for establishing collaboration was not described in the convention. The Bonn Guidelines were adopted in 2002 to support collaboration negotiations. The prior informed consent procedure (PIC) and mutual agreed terms (MAT) are key elements for ABS. For the first time, the benefits in terms of

Fig. 3.4 NP meeting, Nagoya, Japan, 2010.Source: Katsuhiko Ando (Personal communication)

monetary and non-monetary payment were mentioned in the guidelines. The Nagoya Protocol on 12 October 2014 represented internationally significant progress on ABS (Fig. 3.4). In light of the CBD, the Nagoya Protocol provides an international mechanism developed to elaborate and negotiate based on ABS. The Nagoya Protocol consists a total of 36 articles and includes 2 annexes. To encourage technology transfers through access to genetic resources, the Nagoya Protocol draws and highlights the following important aspects of the negotiation: (1) ABS procedures are initiated through a clearing house from each party as a national focal point (Articles 13 and 14); (2) ABS must be in compliance with domestic legislation and regulatory requirements (Articles 15 and 16); (3) the appropriate capacities in terms of manpower, facility, and policies are required for negotiation and technology transfer (Article 22); (4) technology transfer is encouraged (Article 17); and (5) ABS content and concept of benefit in terms of monetary and non-monetary payments are clarified (Article 5 and Annex I).

After the Nagoya Protocol on ABS was adopted in October 2014, working with genetic resources and associated data has required greater attention. Biologists must ensure that they have a legal understanding of how they can and cannot use the genetic resources in their research (Smith et al. 2017a). However, at the moment, only 100 countries have adopted the NP, with 98 countries abstaining (https://absch.cbd.int/).

3.4.2 *ACM: The Asian Consortium for the Conservation and Sustainable Use of Microbial Resources as a Case Study of Asian Collaboration in Culture Collection in Light of the Nagoya Protocol on ABS*

3.4.2.1 ACM Establishment and Activities

Asia covers an area of 44,579,000 square kilometers and is notable for its overall large size and population but also its dense and large settlements. It also has vast barely populated regions, within the continent of 4.4 billion people, which represents 59% of the world's population. Asia contains temperate to tropical climates. It has a diversity of fermented foods from megadiverse countries, such as China, India, Indonesia, Malaysia, and the Philippines (https://en.wikipedia.org/wiki/Megadiverse_countries). Asian countries have a high rate of economic development, with a 5% average growth rate in the annual gross domestic product compared with 2.5% for the world. The protection, conservation, and sustainable utilization of biodiversity have been gained much attention in Asian countries. Collaborations among mBRCs were established in 2004 by representatives of 12 Asian countries (Cambodia, China, Indonesia, Japan, Korea, Laos, Malaysia, Mongolia, Myanmar, the Philippines, Thailand, and Vietnam). To facilitate international collaborations among mBRCs and encourage biotechnology utilizing microbial resources in Asia, the ACM aims are as follows: (1) establishment of a framework for international cooperation to encourage microbiological research, (2) construction of mechanisms for academia and industry to utilize microbial resources, (3) establishment of the Microbial Resource Centre network, (4) establishment of international standards for biological material transfer and benefit sharing, and (5) improvement and sharing of standardized techniques. The ACM focused on three task forces: (1) Asian BRC Network, (2) Human Resource Development, and (3) Management of Material Transfer. Through task force activities, much progress has been made to increase the capacities of ACM members in mBRC management, manpower development, and biotechnology. To date, about 100 international workshops and technical training courses have been conducted by experienced international scientists inside and outside of Asia to update the knowledge and techniques of thousands of young Asian scientists in microbiology, culture collection management, taxonomy and identification, and bioinformatics. Under the ACM umbrella, a number of international bilateral and internal collaborations have been established for technology transfer among ACM member countries (Fig. 3.5). There is, however, a large gap between the advanced mBRCs in Japan, China, and Korea and those of some developing countries, such as Vietnam, Laos, Cambodia, Myanmar, and Mongolia. To encourage technology transfers, many scientists are involved in exchange programs or specific research projects. In accordance with the CBD and Nagoya Protocol, the task force team on the Management of Material Transfer has developed the Network of International Exchange of Microbes under ACM, a mechanism for microbial

Fig. 3.5 ACM 1 meeting, October 2004, Tsukuba, Japan. Source: Katsuhiko Ando (Personal communication)

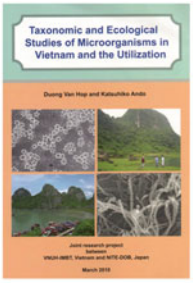
strain transfer and distribution to end users for non-commercial research use (Ando et al. 2014).

3.4.2.2 A Case Study on Bilateral Collaboration Between the National Institute of Technology and Evaluation (NITE), Japan, and the Institute of Microbiology and Biotechnology (IMBT), Vietnam National University, Vietnam

Biotechnology is a priority field in Vietnam policies on science and technology. The conservation of microbial diversity is recognized to be important for the various fields of the life sciences, such as agriculture, food safety, healthcare, and environment. The Vietnam Ministry of Science and Technology has been implementing the national program on the conservation of plants, animals, and microorganisms since 1997. The government ratified both the CBD (1994) and Nagoya Protocol (2014), but the manpower and policy levels need much improvement for internal and external collaboration. In 2004, NITE and IMBT implemented a collaborative project on taxonomic and ecological studies of microorganisms in Vietnam and their utilization. The project was conducted based on the following agreement on common interests of each party: (1) IMBT's need to establish a Vietnam Type Culture Collection (VTCC) and strengthen its ability to study microbial diversity and management and (2) NITE's interest in culture collection enrichment, studying tropical microbial diversity in Vietnam and performing Network of International Exchange of Microbes under ACM mechanisms for microbial strain exchanges, transfers, and distributions to end users for non-commercial research use. After a 10-year implementation period, the project was fruitful for both parties.

Technical training course at IMBT, Hanoi

IMBT scientist studying at NITE, Japan



Report on taxonomic and ecological studies of microorganisms in Vietnam and their utilization

Fig. 3.6 The activities of the collaborative project

The benefits to the IMBT from the collaboration included a great improvement in the capacity to study and manage microbial resources (Fig. 3.6). This is illustrated by the following points:

1. The VTCC is a national mBRC, holding ~10,000 microbial cultures, and 3600 strains are available from an online catalogue (http://vtcc.imbt.vnu.edu.vn/). The VTCC became a member of WFCC in 2008 and registered 2082 cultures in the Global Catalogue of Microorganism in 2013 (http://gcm/wfcc.info). The final report of the project is the first report on the biodiversity of microbes in Vietnam.
2. Technology transfer and the training of young Vietnamese scientists. During the project period (2004–2015), ~20 young IMBT scientists and 30 experienced Japanese scientists were involved in exchange programs in Japan and Vietnam. Through those activities, IMBT staff learned updated techniques, methods, and knowledge in studying microbial diversity and culture collection management; in the isolation, preservation, identification, and taxonomic studies of common microbes (filamentous fungi, actinomycetes, bacteria, yeast, and microalgae); and bioinformatics. In addition, both NITE and IMBT co-conducted 18 workshops and technical training courses in Hanoi that were attended by 150 participants from many institutes and universities in Vietnam.
3. Improved capacity of the VTCC. By implementing the project, the capacity of the VTCC is much improved. Now, the VTCC can extend its activities beyond preservation to the identification and taxonomic study of microbial isolates. At the current time, the VTCC is conducting some R&D activities on screening useful strains for the establishment of a Vietnamese database of cultures with valuable information on stored microbes. Implementing the project was a good case study and allowed in the cataloguing of microbial resources under the CBD in Vietnam. The practical experience gained was useful for amending current Vietnam policies and regulations under the CBD and Nagoya Protocol for worldwide collaboration.

The benefits to NITE from the collaboration were as follows:

1. NITE scientists gained a better understanding of the diversity of tropical microorganisms and their roles in diverse fermented food processes in Vietnam. In total, ten manuscripts written by co-authors from NITE and IMBT were published internationally, and six manuscripts are in progress.
2. By implementing the Network of International Exchange of Microbes under ACM mechanisms, NITE enriched its stored isolates with tropical strains from Vietnam, which are potential genetic sources for R&D.
3. The collaborative project was a good case study for NITE to better understand user demands and related existing policies and regulations. It may be useful for further long-term collaboration in biotechnology with developing countries.

The CBD and Nagoya Protocol identified the sovereign rights of the original country over genetic resources and procedures and established principles for ABS in terms of monetary and non-monetary benefits as practical platforms for collaboration between users and providers. However, successful negotiations based on common interests for collaboration regarding accession and material transfers of microbial resources are not so easy. The concept of fair equitable benefit sharing is quite sensitive. The NITE–IMBT collaborative project was successful and fruitful for both parties. The achievements were not limited to increasing the VTCC's capacity but impacted related legislation in Vietnam to better negotiate collaborations. The key element for this collaboration was that it required being "translated" into the detailed and clear interests of each party in compliance with international and national policies and fruitful negotiations based on a practical understanding.

3.5 Conclusion

The demand for biotechnology and bioindustry development needs more public attempts on studying microbial diversity and its roles in life science. The number of some thousands of new species of microbes found annually is a strong indicator for the attention from scientist's community. The useful roles of microbes in life science development has been discovered.

For any life science activities related to microbes, the quality of the cultures as the starting genetic material is the most important. It is strongly recommended to a new established mBRCs to follow crucial guidelines from WFCC and to update knowledge, techniques, methods, and experiences appropriate to the maintained cultures in culture collection management and operation from long history of experienced mBRCs such as ATCC (USA), CABI, (UK), DSMZ (Germany), and NBRC(Japan).

Promoting international collaboration among mBRCs is in need for life science development. The collaboration is not only limited in information, techniques, methodology, and education but also extended to the exchange in the microbial genetic source. Still there are some differences in regulations in national management of genetic resources from country to country based on particular common

interest. However, CBD and NP are excellent platforms for developing bilateral and multilateral collaborations between and among mBRCs.

Acknowledgments We thank Lesley Benyon, PhD from Edanz Group (www.edanzediting.com/ac), for editing a draft of this manuscript.

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