

Chapter 8

Maintaining Microbial Diversity



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Maintaining Microbial Diversity

HIGHLIGHTS

- Micro-organisms provide benefits and harbor danger. But safeguarding a diversity of these organisms remains important, for few have been cataloged and the potential contribution to agriculture, industry, and medicine is therefore unknown.
- The most cost-effective way to preserve economically important micro-organisms today is through offsite collections. Micro-organisms that are isolated and identified can be stored from a few days to as long as **30** years.
- Technologies used are freeze-drying (the most common method), ultra-freezing (which costs more in labor and materials), immersion in mineral oil, low-temperature freezing, and desiccation. The last three methods are suitable for short-term storage only.
- A high priority in efforts to maintain a diversity of micro-organisms is the need for an integrated database of current collections. Also needed is research on microbial ecology, to better understand the extent to which plants and animals depend on bacteria and fungi to survive.

OVERVIEW

Micro-organisms constitute a vast, though largely unseen, part of the biotic world. Although most frequently discussed in terms of their harmful effects on humans, they are essential to the proper functioning of ecosystems as well as to the survival of many species of plants and animals (19,25) (see box 8-A). The public is less concerned, however, about potential losses of microbial diversity than about plant, animal, or ecosystem diversity (19).

What Is Microbial Diversity?

The wide range of micro-organisms not typically classed as plants or animals includes bacteria, cyanobacteria (blue-green algae), fungi (including yeasts), protozoa, and viruses (see table 8-1). Although the microscopic, single-celled bacteria are generally considered synonymous with the term micro-organism, the field in-

cludes such different things as the large marine algae of ocean kelp beds and the sub-microscopic viruses that infect humans, animals, plants, and other micro-organisms. Even smaller than viruses are those infective agents, such as viroids, that have been found to be nothing more than pieces of genetic material, lacking even the typical protein coats of a virus. Thus, the diversity of micro-organisms is immense, with only a relatively small fraction of micro-organisms having been identified (19,25),

The concept of a species, borrowed from animal and plant biology, cannot be easily applied to all micro-organisms (5,19,25,29). Research frequently focuses on populations of microbial cells that share common nutritional, chemical, or biochemical characteristics. Such populations, each typically descended from a single

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Table 8“1.—Summary of the Characteristics, Problems, and Uses of Micro-Organisms

Organisms	Characteristics	Problems	Uses
Bacteria	Single-cells; spherical rod and spiral forms. Most are saprophytes (use dead matter for food), although some bacteria are photosynthetic.	Some cause disease in humans, animals, and plants,	Break down organic matter and assist soil fertility, waste disposal, and biogas production; source of antibiotics and other chemicals,
Fungi	Variety of forms; microscopic molds, mildews, rusts, and smuts; larger mushrooms and puffballs,	Rot textiles, leather, harvested foods, and other products; cause important plant and animal diseases.	Assist in recycling complex plant constituents such as cel lulose; mushrooms and yeasts important as foods; many used in chemical and pharmaceutical industries.
Algae	Single cells, colonies, or filaments containing chlorophyll and other characteristic pigments; no true roots, stems, or leaves; aquatic.	Cover pond surfaces, producing scum and unpleasant odor and taste (in drinking water); absorb O ₂ from ponds and some produce toxins.	Red and brown seaweeds are important foods in Asia and Polynesia; red algae produce agar; important food source for many ocean fish.
Protozoa	Single cells, or groups of similar cells, found in fresh and sea water, in soil, and as symbionts or parasites in man, animals, and some plants.	Responsible for serious human and animal disease (e. g., malaria, sleeping sickness, dysentery).	Assist in breakdown of organic matter such as cellulose in ruminant nutrition,
Viruses	Infective agents; capable of multiplying only in living cells; composed of proteins and nucleic acids.	Cause variety of human, animal, and plant diseases (e. g., influenza, AIDS, rabies, mosaics).	Important as carriers of genetic information; some infecting pests can be used as biological control agents.

SOURCE: National Academy of Sciences, *Microbial Processes Promising Technologies for Developing Countries* (Washington DC National Academy Press, 1979)



Photo credit: G.E. Pierce and M.K. Mulks

Pseudomonas putida, a bacterium capable of degrading hydrocarbons, is one type of micro-organism.

cell, are termed strains (7). Individually identifiable strains of micro-organisms are thus often regarded as the basic units of microbial diversity.

Micro-organisms are found in nearly all environments (8). Bacteria, for example, have been found living in deep-sea steam vents at temperatures of 3500 C (22). Although some micro-organisms are widely distributed, others may be restricted to a narrow ecological range. Most micro-organisms are found as parts of complex microbial communities or as integral parts of larger ecosystems. Many of these micro-organisms cannot be isolated and grown under controlled laboratory conditions (25).

Status of Microbial Diversity Onsite

Assessing the status and changes in microbial diversity onsite can be difficult. As noted earlier, few micro-organisms have been isolated

and described or identified (25). Therefore, any changes that occur cannot be determined as temporary or permanent.

For example, the composition of microbial populations within environments can be dramatically altered by pollutants (18,19,32). Studies of microbial populations at a pharmaceutical dump site in the Atlantic Ocean indicate that the survival and growth of certain marine micro-organisms over others in the population occur as a result of pollution (27). Although it is clear that pollution or environmental disturbance can produce quantitative changes, definitive evidence of extinction of micro-organisms, as is seen in plants and animals, is rare. But it would seem likely that where micro-organisms are highly adapted to a specific environment, loss of that environment could result in extinction of the micro-organisms (10).

one group of micro-organisms for which the potential for loss has been a particular concern is the macrofungi—more specifically, the edible wild mushrooms. Morels, chanterelles, and other mushrooms have long been collected by fanciers, particularly in the Northwest United States (33). However, increased collection to serve a growing commercial demand for wild mushrooms has raised fears that the most sought-after species could become rare or extinct (1,33). Scientists currently disagree about whether this is possible, for it is not even known

if wild mushrooms can be overharvested (33). In the absence of information, some efforts have been made to limit collection (33). Research on the biology and ecology of these mushrooms and their distribution is needed.

Microbial Diversity Offsite

The principal repositories for those few micro-organisms that have been isolated are offsite collections. Offsite collections of micro-organisms provide easier and quicker access to specific strains than repeatedly returning to onsite sources to obtain them. In addition, it may not always be possible to obtain the same micro-organism from the same place. The fungus from which penicillin was derived, for example, could not again be isolated from air or dust samples in the laboratory where it was first found as a culture contaminant. Offsite collections also are used as reference standards for taxonomic and comparative studies. In microbiology, a “type” strain of a micro-organism is maintained for use as a reference and as a source for subsequent studies (17). It would be impossible to isolate type-strains from natural environments each time comparative studies were initiated (19,21).

Current offsite collections of micro-organisms are actively used as resources by industry and by the scientific community. Yet such collections are rarely established or maintained for preserving microbial diversity (26).

MAINTENANCE TECHNOLOGIES

Onsite maintenance is the only feasible long-term method for maintaining the major portion of microbial diversity, because most of the micro-organisms in any single environment have yet to be identified (19). But existing programs to maintain animal and plant diversity will likely cover all but a few specialized environments (e. g., deep-sea steam vents), so establishing reserves specifically for maintaining micro-organisms should not be necessary,

The most cost-effective approach to providing ready access to the many economically,

medically, agriculturally, or scientifically important micro-organisms today is to preserve them in offsite collections (19). The following sections assess the techniques required to maintain micro-organisms offsite.

Isolation and Sampling

Isolation of micro-organisms and their incorporation into a collection generally reflects a particular set of goals. Laboratory applications, such as those involved in genetic engineering, require specific strains of micro-organisms that,

once obtained, are kept as pure cultures. Micro-organisms have been isolated to study their interrelationships and the way the dynamics between populations influence the entire biological food chain. Some collections represent sampling of specific taxonomic classes of micro-organisms of economic or agricultural importance. The actinomycete collection of the Battelle-Kettering Laboratory (Yellow Springs, OH) and the many collections of varying sizes of Rhizobiurn, the nitrogen-fixing bacteria of legumes, are examples of goal-directed collections. The pathogenic characteristics of a micro-organism, as in the case of a disease-producing virus, can also merit spending funds, time, and expertise to isolate it.

Isolated pure cultures of micro-organisms are necessary for detailed study (7). For some, such as the fungi, a sterile culture of spores on a specially prepared medium may be all that is necessary to obtain a pure culture. Nutritional requirements for various fungi can, however, be specific and difficult to determine. Most bacteria must be cultured on a variety of media that will stimulate growth of possible contaminants, from which pure culture can then be obtained. Viruses are frequently isolated from infected cells or tissues by centrifugation or filtration techniques that separate them from other cellular components. For micro-organisms that consist only of a small piece of genetic material, such as viroids, the newly developed technologies for isolating, multiplying, and characterizing DNA and RNA have been important. The critical determination that an isolated micro-organism is pure can be a lengthy process of repeated culture or separation under varying conditions and can be a research problem in itself (7).

Studies of microbial ecology and microbial diversity are limited by the inability of scientists at the present time to isolate many micro-organisms (19). Identification, for example, generally requires growth in pure culture to allow for nutritional and physiological testing. It is not currently possible to acquire a knowledge of the total microbial diversity in any one environment in a readily definable time period because of this inability to isolate, culture, and

characterize every (or even most) micro-organism present. Thus, sampling of diversity is limited to those micro-organisms for which isolation and culture technologies are available.

Microbial Identification

Identification of isolated micro-organisms can be a lengthy and complex task (for details of the principles and procedures, see ref. 15). Preliminary identification involves standard staining procedures and microscopic examination. Analysis of the results of these initial examinations requires extensive knowledge of micro-organisms and the general characteristics of various taxonomic groups. Information regarding the source of the isolate can also play an important role at this stage.

Following preliminary identification, the micro-organism is subjected to more detailed analysis, frequently consisting of examination of growth characteristics on varying substrates and under varying environmental conditions. These tests establish specific physiological characteristics that aid identification. The general protocol is to work with a pure culture and, using selected tests, narrow the range of possibilities. Once identified, the isolate is then compared to a reference sample using selected diagnostic tests (24).

Biochemical analysis of proteins and DNA, as described for plants (see ch. 7), has been used for identification of many strains of micro-organisms (7). Although these technologies routinely identify the micro-organisms used in research laboratories, they are not generally applied in offsite collections. As the field of genetic engineering has developed, however, the capacity to study, compare, and identify the genomes of micro-organisms has improved (10,31). Such techniques could greatly enhance assessment of diversity and facilitate identification of micro-organisms in offsite collections.

Storage of Micro-Organisms

The purpose of preserving micro-organisms is to maintain a strain for an indefinite period in a viable state. The continuous culture of a

micro-organism is one way to present it, but it is expensive both in materials and in labor and does not ensure that the genetic stability of the micro-organism will be maintained. Continuously subculture organisms can adapt to the specialized conditions of the laboratory and take on characteristics different from those for which they were originally isolated. Long-term storage techniques that minimize such effects have been developed.

Storage of micro-organisms involves reducing metabolic rates and, thus, the rate at which micro-organisms multiply and use nutrients (19). All methods that reduce metabolic rates cause loss of a certain percentage of the sample. Methods need to be developed, therefore, that not only reduce metabolic rates but also prevent decline in viability in order to prevent loss of the strain.

An additional time-consuming but crucial task associated with storage technologies is authentication (19). This task involves the maintenance of accurate records about the culture history and diagnostic characteristics of the strains in a collection. It also involves periodically recovering and culturing stored organisms to determine their viability and to confirm purity and genetic stability.

The majority of micro-organisms currently preserved in culture collections are held by freeze-drying or by ultra-freezing (cryogenic storage) (16,19). These two methods permit storage for extremely long periods of time (currently as long as 30 years) (16). Other special methods for storage of micro-organisms are immersion in mineral oil, low-temperature freezing, and desiccation (16).

Freeze-Drying

Freeze-drying, or lyophilization, is now the most commonly used storage technique for culture collections. Healthy microbial cells, grown under optimal conditions, are dispensed in small, sterile vials or ampules at a relatively high concentration (e. g., 10^6 to 10^7 cells per milliliter of solution). The vials are then quickly frozen in a super-cooled liquid solvent bath or in a mechanical ultra-freezer (at -60°C), and

these frozen suspensions are placed under vacuum to remove the water in them. The vials are then heat-sealed under vacuum by melting the glass tops with an air-gas torch and stored at temperatures lower than 50°C . Lower storage temperatures (-30° to -70°C) may result in lengthened viability.

Chemical agents (cryoprotectants) that protect cells from damage caused by ice-crystal formation during the initial freezing are commonly added to cells before freeze-drying. The American Type Culture Collection (ATCC) routinely uses 10 percent skim milk or 12 percent sucrose for such purposes. Curators at the Northern Regional Research Laboratory of the USDA's Agricultural Research Service, in contrast, prefer bovine or equine serum as a cryoprotectant for all microbial species (19).

Recovery of the lyophilized cells is simple and straightforward. The sealed vial is opened by scoring, and a small amount of liquid-nutrient medium is added to rehydrate the cells. The contents, once rehydrated, are transferred to a culture vessel containing a medium appropriate for growth.

The initial cost of equipping a laboratory to undertake lyophilization is as much as \$25,000 (11). The expense of actually preparing lyophilized cultures, however, is low. The long-term viability of such materials is excellent, and this procedure is thus probably the most cost-effective means of microbial preservation in use today (19). Unfortunately, some microbial species do not fare well under these techniques, and other storage methods must be used,

Ultra-Freezing

Fastidious microbial species (i.e., those with complex nutritional requirements) that do not retain viability under other preservation methods (e.g., plant pathogenic fungi) frequently can be preserved by ultra-freezing (2,19). In this procedure, cells sealed in vials or ampules are frozen at a slow cooling rate (1°C per minute) until they reach -1500°C . The vials are then stored at -1500 to -196°C using liquid nitrogen freezers.



Photo credit W H Siegel

Cells being dispensed into ampules to be frozen and stored in liquid nitrogen (-196°C). The cabinet contains only sterile, filtered air to lessen the chances of contamination of the freeze preparation.



Photo credit W H Siegel

Ampules of freeze-dried or frozen living strains can be stored in mechanical refrigerators at -60°C (-76°F), in walk-in cold rooms at 5°C (40°F), or in vacuum-insulated refrigerators (above) automatically supplied with liquid nitrogen at -196°C (-320°F).

The cryoprotective agents necessary for this procedure differ from the ones used in lyophilization. ATCC routinely uses a mixture of glycerol (10 percent), dimethylsulfoxide (5 percent), and nutrient medium for most bacterial strains. These chemicals are taken into the cells and protect the internal membranes from injury caused by freezing.

Cells stored at cryogenic temperatures must be handled carefully when being recovered. Ice crystals can form as vials are warmed and can kill cells that would otherwise survive the technique. Loss of viability is minimal when the sample is thawed rapidly. Sealed vials are thus put in water at 37°C until all ice melts. Then they are opened and the contents transferred to nutrient medium.

Ultra-frozen cultures must be maintained at very low temperatures at all times during storage. Liquid nitrogen freezers are therefore needed. Proper precautions are important to ensure that such freezers operate properly and have sufficient supplies of liquid nitrogen coolant over long periods of time. Thus, the technique can cost more than freeze-drying, both in labor and in materials necessary to maintain storage temperatures. Ultra-freezing is reserved for microbial species that are not amenable to other, less costly procedures.

Other Methods

Microbial cell cultures can be stored for short periods of time if the culture is overlaid with sterile mineral oil. The oil prevents dehydration and reduces the metabolic rate of the organisms (16). Cells are grown on either nutrient gels or in broth cultures. After incubation and growth, mineral oil is added to the culture to a depth of about 2 centimeters. The cultures are then stored at approximately 40°C . Recovery is by procedures similar to those used for routine subculture. Although cultures preserved in this way have remained viable for as long as 3 years, the method is not considered appropriate for long-term storage of micro-organisms, because cultures have to be recovered, authenticated, and restored every few years (19).

A few micro-organisms, like some of the actinomycetes and some soil-borne spore-forming bacteria, can withstand normal freezing processes and retain both viability and genetic stability. Cultures are stored on a nutrient medium at 0° to -20°C . Cells may remain viable for as long as 2 years, but damage by ice crystal formation is thought to be extensive (19). For

certain microbial cells, low-temperature freezing is inexpensive and useful for a short period. Like immersion in mineral oil, however, repetitive recovery, authentication, and restorage make this technique too labor-intensive for long-term maintenance of micro-organisms.

The majority of microbial cells die if they are dried at ambient temperatures (16). But a few can withstand dehydration and remain viable for moderate periods of time. Spore-forming bacteria are particularly suited to this method of storage. Microbial cells are usually transferred to a sterile, solid material, and then de-

hydrated under vacuum. A soil, paper, or ceramic bead medium is frequently used. Cells also may be suspended in gelatin solution and then drops of the gelatin dried under vacuum. Once dehydrated, the cells must be stored in desiccators but will remain viable longer if refrigerated. Recovery of the cells is by dehydration with nutrient medium and subculture. This method is relatively inexpensive and routinely used for some important bacterial genera (e. g., *Rhizobiurn*). However, other techniques, if available, are preferred for long-term storage (19).

NEEDS AND OPPORTUNITIES

No major technical constraints limit the storage and use of micro-organisms in the United States and most other industrial nations, although developing countries lag behind in the application, use, and technological knowledge of micro-organisms (28). The most satisfactory long-term storage techniques for micro-organisms are also those that are technologically the most sophisticated. In the case of cryogenic storage, the technique requires a dependable source of liquid nitrogen. Lyophilization of cultures in sealed, evacuated glass-ampoules creates culture units with excellent longevity, even when stored at room temperature. The attraction of this method for developing countries is diminished slightly by the relatively high initial cost of equipment and problems keeping such equipment operational (19).

Improvisation in the laboratories of developing-country scientists has resulted in a wide array of variations of standard preservation methods (19). These modified methods are, in many cases, satisfactory to the individual collection curators, though most require micro-organisms to be regularly subculture. This requirement makes these methods suitable only for relatively small collections, and it increases the likelihood of strains becoming genetically adapted to culture and losing their original characteristics.

Catalog Collections

Maintaining and distributing a current catalog is the goal of virtually every collection curator. Without such a compilation to provide potential users with ready access to its contents, the value of a collection is greatly diminished. That very few collections are adequately cataloged is a reflection of just how onerous this task can be. In a sense, this aspect of collection management has been constrained by lack of an appropriate technology (19). The advent of microcomputers and highly adaptable, user-friendly database management systems software heralds a new era enabling a curator to compile, print, and update a catalog inexpensively and with relative ease (9). Such electronic catalogs would make current collections more accessible and improve their management (4).

One way to catalog the contents of various collections is through creation of a National Microbial Resource Network. Two main obstacles can be anticipated to such a network:

1. the differences in history, traditions, and independence of existing collections; and
2. the difficulty of standardizing technical and informational protocols to assure meaningful interchange of germplasm and data among participating network institutions.

One network of microbiological resource centers (MIRCENs) has been addressing these obstacles for almost a decade (12) (see ch. 10). In practice, however, this endeavor has been frustrated by an inability to come up with standardized data sets that reconcile the different orientations of individual collections worldwide and by financial resources that fall far short of the level required (12).

A more focused attempt to achieve an integrated microbial resource database was initiated in 1984 by UNESCO. This MIRCEN project is still being developed and provides for standardization of a minimum data set for characterized strains of *Rhizobium* (the bacteria involved in nitrogen fixation in soybeans, alfalfa, beans, and other legumes); adoption of compatible database management systems; and periodic publication of an integrated catalog of the collections held at Beltsville, Maryland (USA), Porto Alegre (Brazil), Nairobi (Kenya), and Maui, Hawaii (USA).

An appraisal of the lessons learned in the international MIRCEN effort could greatly benefit establishment of a National Microbial Research Network. Reservations may be expressed about whether the institution-building rationale for the MIRCEN program will mean the collections are of less-than-optimal quality; nevertheless, with limited financial resources, the MIRCEN program has achieved a high degree of network effectiveness, including regular global and regional newsletters, electronic data exchange, and computer conferences,

Develop Methods for Isolation and Culture

An important challenge to maintaining micro-organisms offsite is the development of methods for culture of those organisms that have not yet been isolated in the laboratory (19). Basic research into the isolation and cultivation of these fastidious micro-organisms is essential to further applications of the world's microbial diversity. Research on microbial culture would allow better characterization of diversity in natural environments as well as enable more efficient handling of difficult micro-

organisms in existing collections. Efforts to isolate the *Legionella* micro-organism or the retrovirus (human T-cell leukemia-lymphoma virus) associated with acquired immune deficiency syndrome (AIDS) illustrate both the difficulty and importance of such research.

An appreciation of the complexity of the technical barriers faced by microbiologists trying to isolate and culture many micro-organisms is necessary to support research. Basic studies of microbial physiology, through grant programs and in-house research by such agencies as the National Science Foundation, U.S. Department of Agriculture, and the National Institutes of Health (NIH), can improve present abilities to isolate and culture micro-organisms.

Study of Microbial Ecology

Another research priority is that of microbial interactions that permit efficient functioning of the microbial flora of an environment and, ultimately, support higher organisms in that environment. Research into microbial ecology is an integral part of any strategy to preserve micro-organisms. Present understanding of microbial ecology and the extent of microbial diversity in ecosystems is, however, inadequate (19,25). Many plants and animals depend on bacteria and fungi in the environment to survive (25). In some cases, such as digestion in the termite or dairy cattle, microbes are important parts of the organism's basic physiology. Study of the soil micro-organisms that are active in nutrient recycling, such as those associated with nitrogen fixation, are of great potential importance to agriculture.

Grant programs and in-house research at agencies such as NIH, USDA, the Department of Energy, and the Environmental Protection Agency could focus on improved understanding of microbial ecology. Present efforts are spread over several agencies with little coordination. Examination of the overall efforts relating to microbial ecology and diversity could lead to better coordination of research and development of a specific funding program within one agency that would address microbial ecology research.

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