

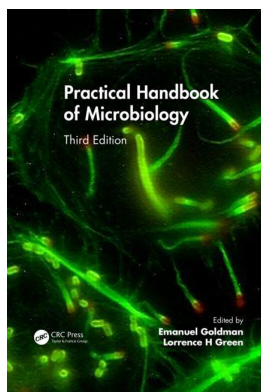
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### **Culturing and Preserving Microorganisms**

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# 3 Culturing and Preserving Microorganisms

*Lorrence H. Green*

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## INTRODUCTION

The use of culture as a diagnostic technique will be described in Chapter 10. It is the purpose of this chapter to discuss only the principles of enrichment culture. This is a technique that has been in use for more than 100 years.<sup>1</sup> It consists of incubating a sample in a medium that encourages the growth of an organism of interest while inhibiting the growth of others. In this way, it can assist the technician in isolating pure colonies of microorganisms from mixtures in which the organisms represent only a very small percentage of the overall flora, and in which isolation by streaking may not be practical. In some cases, as in the isolation of salmonella, first growing a sample in an enrichment culture is a necessary step to increase the small relative number of organisms usually found in the primary sample.<sup>2</sup> While the use of enrichment broths in food and environmental microbiology has been demonstrated, their use in clinical microbiology has not fully been established.<sup>3</sup>

## DESIGNING AN ENRICHMENT MEDIUM

While the media used to enrich for specific microorganisms might differ greatly from each other, they should all contain an energy source, a carbon source, and a source of major and trace elements. Factors such as pH, temperature, and oxygen tension should be appropriate to enhance the growth of

the microorganism of interest.<sup>1</sup> In addition to providing components that select for specific microorganisms, enrichment media may also contain antibiotics or other compounds that inhibit the growth of microbiological competitors. In the case of autotrophic microorganisms such as cyanobacteria or algae, enrichment media may lack the carbon sources required by heterotrophic microorganisms. Recently, it has been shown that the interaction between microorganisms that are jointly selected for by an enrichment media may also affect the efficacy of the media.<sup>4,5</sup>

### ENERGY SOURCE

Most microorganisms are heterotrophs and, as a result, obtain their energy by ingesting organic molecules. When these molecules are broken down, the energy contained in the chemical bonds is liberated.<sup>6</sup> Some microorganisms are autotrophs and are capable of obtaining energy either by oxidizing inorganic chemical compounds (chemoautotrophs) or by directly utilizing light (photosynthetic).<sup>6</sup> Chemoautotrophs derive energy from reduced inorganic molecules or ions, which can include  $H_2$ ,  $NH_4^+$ ,  $Fe^{2+}$ , and  $S^0$ .<sup>1,7</sup> Photosynthetic microorganisms derive their energy directly from light sources, with bacteria and photosynthetic eukaryotes favoring light in the red portion of the spectrum, while cyanobacteria favor light in the blue portion.<sup>1</sup>

### SOURCE OF CARBON

Since autotrophic organisms derive their energy from light, they usually require little more than a source of simple carbon. This can be supplied by adding a solid carbonate or by bubbling  $CO_2$  gas into the media. Heterotrophic organisms can obtain their carbon from a wide variety of sources. The most common of these is carbohydrates. Many of these are actually used as the component parts of biochemical tests used to identify microorganisms. These include carbohydrates (i.e., glucose, maltose, lactose, sucrose), acids found in the TCA cycle (i.e., succinic, oxaloacetic, malic,  $\alpha$ -ketoglutaric), amino acids (i.e., alanine, arginine, glycine, serine), and fatty acids (i.e., lactic, pyruvic, butyric, propionic).

In the past few years, it has been realized that microorganisms capable of using toxic compounds as carbon sources exist. As such, research has focused on enriching samples for microorganisms that can be used for bioremediation of environmental spills. Pant and Pant have recently reviewed the mechanisms by which microorganisms metabolize trichloroethylene, recognized as the most commonly found contaminant at Superfund sites.<sup>8</sup>

More recently, Wu et al. have reviewed the use of periphytons in the bioremediation of surface waters.<sup>9</sup>

### TRACE AND MAJOR ELEMENTS

All microorganisms also require varying amounts of certain elements. Major elements such as nitrogen, potassium, sodium, magnesium, and calcium can be found as salts. These can include chlorides ( $NH_4Cl$ ,  $KCl$ ,  $NaCl$ ), sulfates ( $MgSO_4 \cdot 7H_2O$ ,  $Na_2SO_4$ ,  $(NH_4)_2SO_4$ ), carbonates ( $MgCO_3$ ,  $CaCO_3$ ), nitrates ( $Ca(NO_3)_2 \cdot 4H_2O$ ), and phosphates ( $K_2HPO_4$ ,  $KH_2PO_4$ ).<sup>1</sup> Other elements may also be required in trace amounts. These can include nickel, iron, zinc, manganese, copper, cobalt, boron, molybdenum, vanadium, strontium, aluminum, rubidium, lithium, iodine, and bromine.<sup>10</sup>

### pH

While most organisms prefer to grow in a neutral pH environment, the pH level can be used as an enrichment method. Acidophiles, for instance, can be enriched by lowering the pH in the media.<sup>11</sup>

## PHYSICAL COMPONENTS

Although researchers usually tend to focus on ingredients that can be added to a liquid medium, it should be remembered that manipulating the physical environment that the culture is placed in for temperature, oxygen tension, and even pressure may offer some microorganisms a competitive advantage in reproduction. The levels of these agents will be determined by which organism the technician is trying to isolate.<sup>12</sup> Thermophiles, such as *Thermogladius shockii*, for instance, can be enhanced by growing cultures at temperatures as high as 80°C–95°C.<sup>13</sup>

## ANTIMICROBIAL AGENTS

Enrichment for specific groups or species of microorganisms can not only be achieved by defining conditions that will preferentially allow them to grow but can also be achieved through the use of agents that will inhibit the growth of competing organisms. In some instances, fast-growing organisms can be inhibited, to give fastidious or slower-growing organisms an advantage in a particular sample. An interesting clinical consequence of the use of antimicrobial agents as a method of enrichment is the observation of resistant organisms in patients who have been hospitalized. Hui et al.<sup>14</sup> have found that previous antibiotic exposure of nosocomial patients using mechanical ventilation can lead to an increase in antibiotic resistance.

## PRESERVATION OF MICROORGANISMS

### INTRODUCTION

Many laboratory test procedures require the use of microorganisms as reagents, while advances in biotechnology have resulted in the creation of engineered microorganisms. In both instances, to obtain consistent results, these organisms must be preserved in a manner that will allow for their genetic stability and long-term survival. Preservation of microorganisms can be accomplished by a variety of methods. These can include subculturing them, reducing their metabolic rate, or putting them into a state of quasisuspended animation.<sup>15</sup> The chosen method will usually depend on which organism one is trying to preserve. Despite the introduction of newer methodologies, many of the techniques that are used have not changed much since the previous editions of this book. Most still involve the use of drying, lyophilization, or storage in freezing or subfreezing temperatures.

### METHODS

#### Serial Subculture

This is a simple method in which the cultures are periodically passed in liquid or agar media. Some cultures can be stored on agar media in sealed tubes and survive for as long as 10 years.<sup>16</sup> This method has been used extensively for fungi but usually requires storage under mineral oil.<sup>15</sup> Despite its simplicity and applicability for cultures that cannot survive harsher preservation methods, serial subculture is not a very satisfactory method. In our hands, we have had problems with contamination, culture death, and the unintended selection of mutants. This has been particularly difficult in cases in which we were trying to develop new products for the identification of microorganisms. It was imperative to periodically confirm the identity of the organisms being used in the database.

#### Storage at Low Temperatures

Some species are capable of being stored for long periods at 4°C–8°C on agar plates or on slants. Sorokulova et al. have found that the use of acacia gum in storage media will allow long-term storage of organisms even at room temperature.<sup>17</sup>

In our lab, we have been able to store cultures at  $-20^{\circ}\text{C}$  for extended periods. We have found that this can be accomplished by growing the cultures in liquid media for 24–48 h and then mixing one part sterile glycerol to three parts culture. While this does not work for all organisms, it allows the use of a standard refrigerator for preserving cultures. Many laboratories store organisms at  $-70^{\circ}\text{C}$  to  $-80^{\circ}\text{C}$  by first mixing them with 10% glycerol.<sup>18</sup> Many cultures can be stored indefinitely in liquid nitrogen. A method for this can be found at [www.cabri.org](http://www.cabri.org) (accessed April 1, 2014). While the use of liquid nitrogen is a good method for storing microorganisms, for most laboratories, it requires the expense of constantly refilling a specialized thermos. Tedeschi and De Paoli in their review of cryogenic methods of preserving microorganisms point out that in this era of being able to manipulate the microorganism genome, it is important to pick a cryogenic preservation method that will preserve the genotype and phenotype of the original organism.<sup>19</sup>

### Freeze-Drying

This is a widely used method in which a suspension of microorganisms is frozen and then subjected to a vacuum to sublimate the liquid. The resulting dried powder is usually stored in vials sealed in a vacuum. Many factors can affect the stability of the culture. These include the growth media, the age of the cultures, the phase of the culture, and the concentration of the organisms in the suspension.<sup>15,20</sup> The length of sample viability can vary substantially, but cultures surviving for up to 20 years have been reported.<sup>21</sup> Freeze-drying requires a cryoprotective agent to provide maximal stability. Usually, the organisms are suspended in 10% skim milk.<sup>22</sup>

### Storage in Distilled Water

This was cited as a method of preservation of *Pseudomonas* species and fungi in the first edition of this book.<sup>15</sup> Recent studies have found that it is still an effective method of preserving fungi,<sup>23</sup> as well as a wide variety of bacteria, including *Pseudomonas fluorescens*, *Erwinia* spp., *Xanthomonas campestris*, *Salmonella* spp., *Yersinia enterocolitica*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus*.<sup>24</sup> Stationary-phase organisms grown on agar media and then suspended in 10 mL of sterile water were found to be stable when sealed with parafilm membranes and then stored at room temperature in the dark. Even greater stability could be obtained by suspending the organisms in a screw-capped tube with phosphate buffered saline at pH 7.2, containing  $15.44\ \mu\text{M}\ \text{KH}_2\text{PO}_4$ ,  $1.55\ \text{mM}\ \text{NaCl}$ , and  $27.09\ \mu\text{M}\ \text{Na}_2\text{HPO}_4$ .<sup>24</sup>

### Drying

Sterile soil or sand has been used for preserving spore-forming organisms by adding suspensions and then drying at room temperature. In addition, bacterial suspensions have been mixed with melted gelatin and then dried in a desiccator. Both methods have produced samples that are stable for long periods of time.<sup>15</sup> Recently, a method has been developed in which a microliter quantity of a bacterial suspension is mixed with a predried activated charcoal cloth-based matrix contained within a resealable system that can then be stored. Experiments with *E. coli* have found that viability of over a year at  $4^{\circ}\text{C}$  can be obtained.<sup>25</sup>

## RECOVERY AND VIABILITY OF PRESERVED MICROORGANISMS

Several factors can affect the viability of microorganisms that have been stored by either freezing or drying. These can include the temperature at which microorganisms are recovered, the type and volume of the media used for recovery, and even how quickly microorganisms are solubilized in a recovery medium.<sup>15</sup> Once reconstituted, microorganisms should be evaluated for cell survival.

Usually, the losses of preservation can be overcome by initially preserving large numbers of microorganisms. In doing this, one must be careful to avoid two problems. The first is that if only a very small fraction of organisms is recovered, this can result in the selection of biochemically

distinct strains. The second is that if a culture has inadvertently been contaminated with even a few microorganisms, the preservation technique may lead to a selection for the contaminant.<sup>26</sup>

Drying and freeze-drying have been known to cause changes in several characteristics of preserved microorganisms. These can include colonial appearance and pathogenicity.<sup>15</sup> After revival from a frozen or dried state, many protocols usually advise subculturing the organisms at least two or three times in an attempt to restore any characteristics that may have been lost and to confirm the genetic identity of these organisms.<sup>15</sup>

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