General Microbiology

Methods of studying microorganisms

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Except for certain studies where bacterial populations are examined in their natural habitats, bacteria are usually cultivated and studied under laboratory conditions. Numerous media (singular, medium) have been developed for bacterial cultivation. Because the nutritional requirements of bacteria vary widely, there are great differences in the chemical compositions of the media used in the laboratory. Bacteria also exhibit wide differences with respect to the physical conditions favoring their growth, such as temperature, pH and gaseous environment. The successful cultivation of bacteria requires an awareness of all of these factors.

Nutritional requirements

All forms of life, from microorganisms to human beings, share certain nutritional requirements for growth and normal functioning. The following observations (Table 1) substantiate this statement and also illustrate the great diversity of nutritional types found among bacteria.

Table 1: Nutritional requirement of some heterotrophic bacteria

Bacteria	Inorganic Salts	Organic Carbon	Atmos-pheric N_2	Inorganic Nitrogen	One Amino Acid	Two or more Amino Acids	One Vitamin	Two or More Vitamins
Azospirillum brasilense	+	+	+					
Escherichia coli	+	+		+	+			
Salmonella typhi	+	+		+	+			
Proteus vulgaris	+	+		+			+	
Staphylococcus aureus	+	+		+		+	+	
Lactobacillus acidophilus	+	+		+		+		+

1. All organisms require a source of energy. Some rely on chemical compounds for their energy and are designated as chemotrophs. Others can utilize radiant energy (light) and are called phototrophs. Both chemotrophs and phototrophs exist among bacteria.

2. All organisms require a source of electrons for their metabolism. Some organisms can use reduced inorganic compounds as electron donors and are termed lithotrophs (some may be chemolithotrophs other photolithotrophs). Other organisms use organic compounds as electron donors and are called organotrophs.

3. All organisms require carbon in some form for use in synthesizing cell components. All organisms require at least small amounts of CO_2. However, some can use CO_2 as their major,

or even sole, source of carbon; such organisms are termed autotrophs. Others require organic compounds as their carbon source and are termed heterotrophs.

4. All organisms require nitrogen in some form for cell components. Bacteria are extremely versatile in this respect. Unlike eucaryotes, some bacteria can use atmospheric nitrogen. Others thrive on inorganic nitrogen compounds such as nitrates, nitrites, or ammonium salts, and still others derive nitrogen from organic compounds such as amino acids.

5. All organisms require oxygen, sulfur and phosphorous for cell components. Oxygen is provided in various forms, such as water; component atoms of various nutrients; or molecular oxygen. Sulfur is needed for synthesis of certain amino acids (cysteine, cystine and methionine). Some bacteria require organic sulfur compounds, some are capable of utilizing inorganic sulfur compounds, and some can even use elemental sulfur. Phosphorous, usually supplied in the form of phosphate, is an essential component of nucleotides, nucleic acids, phospholipids, teichoic acids, and other compounds.

6. All living organisms require metal ions, such as K^{+}, Ca^{2+}, Mg^{2+} and Fe^{2+} for normal growth. Other metal ions are also needed but usually only at very low concentrations, such as Zn^{2+}, Cu^{2+}, Mn^{2+}, Mo^{6+}, Ni^{2+}, and Co^{2+}; these are added in trace quantity in culture media which is sufficient to support bacterial growth. Not all the biological functions of metal ions are known, but Fe^{2+}, Mg^{2+}, Zn^{2+} Mo^{6+}, Mn^{2+} and Cu^{2+} are known to be cofactors for various enzymes. Most bacteria do not require Na^{+}, but certain marine bacteria, cyanobacteria, and photosynthetic bacteria do require it. For those members of the archaeobacteria known as the "red extreme halophiles", the requirement is astonishing: they cannot grow with less than 12 to 15 percent NaCl. High level of NaCl maintains integrity and stability of cell wall and enzyme activity.

7. All living organisms contain vitamins and vitamin like compounds. These function either as coenzymes for several enzymes or as the building blocks for coenzymes. Some bacteria are capable for synthesizing their entire requirement of vitamins from other compounds in the culture medium, but others cannot do so and will not grow unless the required vitamins are supplied preformed to them in the medium. Research in bacterial nutrition led to the discovery of some of the vitamins required by humans, and metabolic studies with bacteria contributed to our understanding of how these vitamins are synthesized and how they function.

8. All living organisms require water, and in the case of bacteria all nutrients must be in aqueous solution before they can enter the cells. Water is a highly polar compound that is unequaled in its ability to dissolve or dispense cellular components and to provide a suitable milieu for the various metabolic reactions of a cell. Moreover, the high specific heat of water provides resistance to sudden, transient temperature changes in the environment. Water is also a chemical reactant, being required for the many hydrolytic reactions carried out by a cell.

Nutritional types of bacteria

It is apparent that bacteria can be divided into many groups on the basis of their nutritional requirements. The major separation is in two groups, phototrophs and chemotrophs.

Phototrophs

Among the phototrophic bacteria are species that use inorganic compounds as their source of electrons (i.e. photolithotrophs). For example, *Chromatium okenii* uses H_2S as its electron donor, oxidizing it to elemental sulphur:

$$H_2S \rightarrow S + Ze^- + 2H^+$$

Some phototrophic bacteria are not restricted to being phototrophic. As indicated before, chemotrophs rely on chemical compounds rather than light for their energy, and under some circumstances a phototrophic bacterium can grow as a chemotrophs. For example, in the absence of O_2 (i.e. under anaerobic conditions) *R. rubrum* is dependent on light as its source of energy and lives as a photoorganotroph: however, in the presence of O_2 it can grow in the dark as a chemoorganotroph.

Among the chemotrophic bacteria are species that use inorganic compounds as their source of electrons (i.e. chemolithotrophs). For example, bacteria of the genus *Nitrosomonas* use ammonia as their electron source, obtaining energy by oxidizing ammonia to nitrite:

$$NH_4^+ + {}^{3/2}O_2 + H_2O \rightarrow NO_2^- + 2H_3O^-$$

This reaction involves a net transfer of 6 electrons, causing a valence change of the nitrogen atom from –3 to +3.

Many other chemotrophic bacteria use organic compounds, such as sugars and amino acids, as electron donors and are therefore chemooganotrophs.

Certain bacteria can grow as either chemolithotrophs or chemoorganotrophs. For example, *Pseudomonas pseudoflava* can use either the organic compound glucose or the inorganic compound H_2 as its source of electrons:

$$\underset{\text{glucose}}{C_6H_{12}O_6} + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^-$$

$$H_2 \rightarrow 2H^+ + 2e^-$$

Autotrophs and Heterotrophs

Chemolithotrophic bacteria of the genus *Nitrosomonas* are able to oxidize ammonia to nitrite, thereby obtaining sufficient energy to assimilate the carbon of CO_2 into cell components (CO_2 fixation).

$$CO_2 + 4e^- + 4H^+ \rightarrow [CH_2O] + H_2O$$

where (CH_2O) represents carbohydrate. Organisms that can use CO_2 as their sole source of carbon for assimilation are termed autotrophs.

Until recently it was thought that all chemolithotrophic bacteria were autotrophs. Although this is true for most chemolithotrophs, a few are not recognized as being chemolithotrophic heterotrophs (mixotrophs); i.e. they obtain energy by utilizing inorganic electron donors, but obtain most of their carbon from organic compounds. One such organism is *Desuflovibrio desulfuricans,* which uses electrons from H_2 for the reduction of sulfate, yet derives most of its carbon from organic compounds in the culture medium.

Some autotrophs are facultative autotrophs i.e. they can either live as autotrophs, deriving their carbon from CO_2, or they can live as heterotrophs, deriving their carbon from organic compounds. For example, *P. pseudoflava* can live as a heterotroph using glucose as a source of carbon for assimilation (and also as its source of electrons, as mentioned above); however, if H_2 is provided as the electron source, then it can use CO_2 as its sole carbon source and can grow as an authotroph.

Cultivation of Autotrophs

In terms of chemical complexity of nutrient substances required for growth, the autotrophic bacteria exhibit the simplest requirements (Table 2). Medium which is composed of known chemical compounds, it is called a chemically defined or synthetic medium. The fact that an organism can grow and reproduce in such a mixture of inorganic compounds indicates that it has an elaborate capacity for synthesis. That is, the organism can transform these compounds into the carbohydrates, proteins, nucleic acids, lipids, vitamins and other complex organic substances that constitute the living cell.

Table 2: General growth medium for Autotrophs

$NaNO_3$	1.5
K_2HPO_4	0.04
$MgSO_4.7H_2O$	0.075
$CaCl_2.2H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA (disodium magnesium salt	0.001
Na_2CO_3	0.02
Trace metal mix	1 ml

The trace metal mix contains constituents in g/1

H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.39
$CuSO_4.5H_2O$	0.079
$Co\ (NO_3)_2.6H_2O$	0.0494

Heterotrophs

Heterotrophic bacteria have been studied more extensively than the authotrophs because heterotrophs, in a sense, are of more immediate concern to us: it is here that we find all the species that cause diseases of human beings, other animals, and plants as well as those that constitute the greater part of the microbial population in our immediate environment. This does not mean that autotrophs are less important rather they are of utmost importance in less conspicuous but indispensable processes in nature such as cycling of elements through biological systems.

Cultivation of Heterotrophs: The heterotrophic bacteria, constitute one major nutritional group, vary considerably in the specific nutrients required for growth, particularly with respect to their organic carbon sources, nitrogen sources, and vitamin requirements (Table 3). The requirements may be relatively simple or complex, depending on the species. *E. coli* has much simpler nutritional requirements than Lactobacilli. Organisms such as Lactobacilli that have elaborate requirements for specific nutrients, i.e. vitamins and other growth-promoting substances, are designated fastidious heterotrophs.

Table 3: Medium for cultivation of heterotrophs

$NH_4H_2PO_4$	1 g
Glucose	5 g
NaCI	5 g
$MgSO_4 .7H_2O$	0.2 g
K_2HPO_4	1 g
H_2O	1000 ml

Obligate parasites

Some bacteria have not yet been successfully cultivated on an artificial medium, and their nutritional and physical requirements are not understood. At present, such bacteria can be propagated only in association with a living host which, in a sense, serves as the medium. One example is the bacterium that causes leprosy, *Mycobacterium leprae,* which can be cultivated by infecting mice or armadillos. Other examples include the rickettsias, the chlamydias, and the spirochete that causes syphilis, *Treponema pallidum*.

Chemically defined media

Chemically defined media are needed for the cultivation of autotrophs and are also useful for defining the nutritional requirements of heterotrophs. However, for the routine cultivation of heterotrophs, chemically defined media are not generally used. Instead, certain complex raw materials such as peptones, meat extract and yeast extract are used, and the resulting media support the growth of a wide variety of heterotrophic bacteria. Agar is included as a nonnutritive solidifying agent when a solid medium is desired. Examples of relatively simple liquid and solid media that support the growth of many common heterotrophs are nutrient broth and nutrient

agar. The addition of yeast extract to each of these formulas improves the nutrient quality, since yeast extract contains several of the B vitamins and other growth-promoting substances. Other complex supplements such as bovine rumen fluid, animal blood, blood serum, or extracts of plant and animal tissues may be required for the cultivation of certain fastidious heterotrophs.

Types of media

Many special purpose media are needed to facilitate recognition, enumeration and isolation of certain types of bacteria which may be classified as follows:

Selective media

These media provide nutrients that enhance the growth and predominance of a particular type of bacterium and do not enhance (and may even inhibit) other types of organisms that may be present. For instance, a medium in which cellulose is the only carbon source will specifically select for or enrich the growth of cellulose-utilizing organisms when it is inoculated with a soil sample containing many kinds of bacteria. As an example of a different type of selective medium, the isolation of the gonorrhea-causing organism, *Nesseria gonorrhoeae*, from a clinical specimen is facilitated by the use of media containing certain antibiotics; these antibiotics do not affect *N. gonorrhoeae* but do inhibit the growth of contaminating bacteria.

Differential Media

Certain reagents or supplements, when incorporated into culture media, may allow differentiation of various kinds of bacteria. For example, if a mixture of bacteria is inoculated onto a blood-containing agar medium (blood agar), some of the bacteria may hemolyze (destroy) the red blood cells; other do not. Thus one can distinguish between hemolytic and nonhemolytic bacteria on the same medium.

Assay Media

Media of prescribed compositions are used for the assay of vitamins, amino acids and antibiotics. Media of special composition are used for testing disinfectants.

Media for enumeration of bacteria

Specific kinds of media are used for determining the bacterial content of milk and water. Their composition must be as per the prescribed specifications.

Media for characterization of Bacteria

A wide variety of media are conventionally used to determine the type of growth produced by bacteria, as well as to determine their ability to produce certain chemical changes.

Maintenance Media

Satisfactory maintenance of the viability and physiological characteristics of a culture over time may require a medium different from that which is optimum for growth. Prolific, rapid growth may also be associated with rapid death of the cells at the end of the growth phase. For example, glucose in a medium frequently enhances growth, but acid harmful to the cells is likely to be produced. Therefore, omission of the glucose is preferable in a maintenance medium.

Solid and semi-solid media

In addition to liquid media, solid and semi-solid media are widely used for cultivation of bacteria. Solid media are useful for isolation of bacteria or for determining the characteristics of colonies. The solidifying agent is usually agar, which at concentrations of 1.5 to 2.0 percent forms firm, transparent gels that are not degraded by most bacteria. Silica gel is sometimes used as an inorganic solidifying agent for autotrophic bacteria.

Semi-solid media, prepared with agar at concentrations of 0.5 percent or less, have a soft, custard like consistency and are useful for the cultivation of microaerophilic bacteria for determination of bacterial motility.

Preparation of Media

Some naturally occurring substances are used for the cultivation of bacteria. Notable among these is milk, usually skimmed rather than whole. Such natural materials are merely dispensed into tubes or flasks and sterilized before use. Media of the nutrient broth or nutrient agar type are prepared by compounding the required individual ingredients, or more conveniently, by adding water to a dehydrated product which contains all the ingredients. Practically all media are available commercially in powdered form.

The preparation of bacteriological media usually involves the following steps:

1. Each ingredient, or the complete dehydrated medium, is dissolved in the appropriate volume of the distilled water.
2. The pH of the fluid medium is determined with a pH meter and adjusted if necessary by either dilute acid or alkali.
3. If a solid medium is desired, agar is added and the medium is boiled to dissolve the agar.
4. The medium is dispensed into tubes or flasks.
5. The medium is sterilized, generally by autoclaving. Some media (or specific ingredients) that are heat-labile are sterilized by filtration.

Physical conditions required for growth

In addition to knowing the proper nutrients for the cultivation of bacteria, it is also necessary to know the physical environment in which the organisms will grow best. Just as bacteria vary greatly in their nutritional requirements, so do they exhibit diverse responses to physical conditions such as temperature, gaseous conditions, and pH.

Temperature

Since all processes of growth are dependent on chemical reactions and since the rates of these reactions are influenced by temperature, the pattern of bacterial growth can be profoundly influenced. The temperature that allows for most rapid growth during a short period of time (12 to 24 h) is known as the optimum growth temperature. It should be noted, however, that the optimum growth temperature thus defined may not necessarily be optimum for other cellular activities. On the basis of their temperature relationships, bacteria are divided into three main groups:

Table 4: Characteristics of several species of bacteria with regard to temperatures at which they grow

	Temperature of Growth, ^{o}C		
	Minimum	Optimum	Maximum
Vibrio marinus strain MP-1	-1	15	20
Vibrio psychroerythrus	0	15	19
Pseudomonas fluorescens	4	25-30	40
Staphylococcus aureus	6.5	30-37	46
Corynebacterium diphtheriae	15	37	40
Neisseria gonorrhoeae	30	35-36	38.5
Streptococcus thermophilus	20	40-45	50
Thermoactionormy vulgaris	27-30	60	65-70
Thermus aquaticus	40	70-72	79

1. Psychrophiles

Psychrophiles are able to grow at 0^{o}C or lower, though they grow best at higher temperatures. Many microbiologists restrict the term psychrophile to organisms that can grow at 0^{o}C but have an optimum temperature of 15^{o}C or lower and a maximum temperature of about 20^{o}C; the term psychrotroph or facultative *psychrophile* is used for those organisms able to grow at 0^{o}C which grow best at temperatures in the range of about 20 to 30^{o}C.

During isolation of strict psychrophiles it is usually necessary to maintain the source samples (for example, Antartic soil samples) at cold temperatures from the time they are collected and also to chill all media before attempting isolation. This is because strict psychrophiles usually die if they are even temporarily exposed to room temperature. Even at optimum growth temperatures, it often takes two or three weeks for colonies of psychrophiles to develop.

The physiological factors responsible for the low temperatures maxima for strict psychrophiles are not entirely clear, but some factors that have been implicated are heat instability of ribosomes and various enzymes, increased leakage of cell components and impaired transport of nutrients above the maximum temperature.

2. Mesophiles

Mesophiles grow best within a temperature range of approximately 25 to 40^{o}C. For example, all bacteria that are pathogenic for humans and warm-blooded animals are mesophiles, most growing best at about body temperature (37^{o}C).

3. Thermophiles

Thermophiles grow best at temperatures above 45^{o}C. The growth range of many thermophiles extends into the mesophilic region; these species are designated facultative thermophiles. Other thermophiles cannot grow in the mesophilic range; these are termed true thermophiles, obligate thermophiles, or stenothermophiles.

Factors that have been implicated in the ability to grow at high temperatures are an increased thermal stability of ribosomes, membranes and various enzymes. Loss of the fluidity that exists within the lipid bilayer of the cytoplasmic membrane may be a factor governing the minimum temperature.

It is important to note that a bacterial species may not manifest the same characteristics in every detail when grown at different temperatures. For example, *Serratia marcescens* forms a blood-red to orange pigment when cultured at 25^{o}C but produces little or no pigment when cultured at 37^{o}C. Similarly, *Lactobacillus plantarum* does not require the amino acid phenylalanine for growth when cultured at 25^{o}C but does require it at 37^{o}C.

Gaseous Requirements

The principal gases that affect bacterial growth are oxygen and carbon dioxide. Bacteria display such a wide variety of responses to free oxygen that it is convenient to divide them into four groups as follows:

1. **Aerobic bacteria** require oxygen for growth and can grow when incubated in an air atmosphere (i.e. 21 percent oxygen).

2. **Anaerobic bacteria** do not use oxygen to obtain energy; moreover, oxygen is toxic for them and they cannot grow when incubated in an air atmosphere. Some can tolerate low levels of oxygen (non-stringent or tolerant anaerobes), but others (stringent or strict anaerobes) cannot tolerate even low levels and may die upon brief exposure to air.

3. **Facultatively anaerobic bacteria** do not require oxygen for growth, although they may use it for energy production if it is available. They are not inhibited by oxygen and usually grow as well under an air atmosphere as they do in the absence of oxygen.

4. **Microaerophilic bacteria** require low levels of oxygen for growth but cannot tolerate the level of oxygen present in an air atmosphere.

Oxygen Toxicity

Oxygen is both beneficial and poisonous to living organisms. It is beneficial because of its strong oxidizing ability makes it an excellent terminal electron acceptor for the energy-yielding process known as respiration. However, oxygen is also a toxic substance. Aerobic and facultative organisms have developed protective mechanisms that greatly mitigate this toxicity, but microaerophiles and anaerobes are deficient in these mechanisms and are restricted to habitats where little or no oxygen is present. The following factors are among those that have been implicated in oxygen toxicity.

1. Oxygen inactivation of enzymes: Molecular oxygen can directly oxidize certain essential reduced groups, such as thiol (-SH) groups, or enzymes, resulting in enzyme inactivation. For instance, the enzyme complex known as nitrogenase, responsible for nitrogen fixation, is irreversibly destroyed by even small amounts of oxygen.

2. Damage due to toxic derivatives of oxygen. Various cellular enzymes catalyze chemical reactions involving molecular oxygen; some of these reactions can result in addition of a single electron to an oxygen molecule, thereby forming a superoxide radical (O_2):

$$O_2 + e^- \rightarrow O_2^-$$

Superoxide radicals can inactivate vital cell components. However, recent studies suggest that their greatest detrimental action is through production of even more toxic substances such as hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH-) by means of the following reactions.

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

$$O_2^- + H_2O_2 \xrightarrow{\text{chelated iron}} O_2 + OH^- + OH\text{-}$$

Hydroxyl radicals are among the most reactive free radicals known to organic chemistry and can damage almost every kind of molecule found in living cells. Hydrogen peroxide is not a free radical, but it is a powerful oxidizing agent that is highly toxic to many kinds of cells. Another toxic derivative of oxygen is an energized form known as singlet oxygen ($^1\Delta_g$) O_2, which is produced in biological systems by certain photochemical reactions.

Aerobic and facultative organisms have developed various protective mechanisms against the toxic forms of oxygen. One is the enzyme known as superoxide dismutase, which eliminates superoxide radicals by greatly increasing the rate of reaction above. The hydrogen perodixe produced by this reaction can in turn be dissipated by catalase and perxidase enzymes:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

$$H_2O_2 + \text{reduced substrate} \xrightarrow{\text{peroxidase}} 2H_2O + \text{oxidized substrate}$$

Note that elimination of either superoxide radicals or hydrogen peroxide can prevent the formation of the highly dangerous hydroxyl radicals, since both reactants are required for reaction (3).

In general, anaerobic bacteria have either no superoxide dismutase or only relatively low levels compared to aerobes. Many anaerobes are also deficient in catalase and/or peroxidase. This may help to explain, at least in part, their sensitivity to oxygen, although other factors are probably involved as well.

Cultivation of aerobic bacteria

To grow aerobic or facultative bacteria in tubes or small flasks, incubation of the medium under normal atmospheric conditions is generally satisfactory. However, when aerobic organisms are to be grown in large quantities, it is advantageous to increase to exposure of the medium to the atmosphere. This can be accomplished by dispensing the medium in shallow layers, for which special containers are available. Aeration can also be increased by constantly shaking the inoculated liquid cultures.

Cultivation of anaerobic bacteria

Stringent anaerobes can be grown only by taking special precautions to exclude all atmospheric oxygen from the medium. Such an environment can be established by using one of the following methods:

1. ***Prereduced media***

   During preparation, the culture medium is boiled for several minutes to drive off most the dissolved oxygen. A reducing agent, e.g. cysteine, is added to further lower the oxygen content. Oxygen-free N_2 is bubbled through the medium to keep it anaerobic. The medium is then dispensed into tubes which are being flushed with oxygen-free N_2, stoppered tightly, and sterilized by autoclaving. Such tubes can be stored for many months before being used. During inoculation, the tubes are continuously flushed with oxygen free CO_2 by means of a cannula, restoppered and incubated.

2. ***Anaerobic chamber***

   This refers to a plastic anaerobic glove box that contains an atmosphere of H_2, CO_2 and N_2. Culture media are placed within the chamber by means of an air lock which can be evacuated and refilled with N_2. From the air lock the media are placed within the main camber. Any O_2 in the media is slowly removed by reaction with the H_2, forming water; this reaction is aided by a palladium catalyst. After being rendered oxygen-free, the media are inoculated within the chamber (by means of the glove ports) and incubated (also within the chamber).

Non-stringent anaerobes can be cultured within an anaerobic jar. Inoculated media are placed in the jar along with an $H_2 + CO_2$ generating system. After the jar is sealed, the oxygen present in

the atmosphere within the jar, as well as that dissolved in the culture medium, is gradually used up through reaction with the hydrogen in the presence of a catalyst.

Acidity or alkalinity (pH)

For most bacteria the optimum pH for growth lies between 6.5 and 7.5, and the limits generally lie somewhere between 5 and 9. However, a few bacteria prefer more extreme pH values for growth. For example, *Thiobacillus thiooxidans* has an optimum pH of 2.0 to 3.5 and can grow in a range between pH 0.5 and 6.0. On the other hand, an unclassified bacterium isolated from an alkaline spring in California was found to grow best at a pH of 9.0 to 9.5 and could grow within a range from 8.0 to 11.4.

When bacteria are cultivated in a medium originally adjusted to a given pH, for example, 7.0, it is very likely that this pH will change as a result of the chemical activities of the organism. If a carbohydrate is present it may be fermented or oxidized to organic acids, thus decreasing the pH of the medium. If the salt of an organic acid is supplied as a carbon source (e.g. sodium malate), its oxidation by bacteria will cause an increase in pH. Such shifts in pH may be so great that further growth of the organism is eventually inhibited.

Radical shifts in pH can be prevented by incorporating a buffer (i.e. a substance that resists change in pH) into the medium. A buffer is a mixture of a weak acid and its conjugate base {e.g. acetic acid [CH_3COOH] and acetate [CH_3COO^-]}. Such mixtures have maximum buffering capacity at the pH where the concentration of the acid equals that of its conjugate base. This pH value is called the pKa and is the negative logarithm of the dissociation constant of the acid. Phosphate buffer, i.e. a combination of $H_2PO_4^-$ and HPO_4^{2-} havng a pKa of 6.8 is widely used in bacteriological media. Some of the nutritional ingredients of the medium, such as peptones, also possess some buffering capacity because the component amino acids provide weak acid/conjugate base systems [e.g. –COOH/-COO^-, -NH_3^+/-NH_2, -NH_2^+/-NH). The extent to which a medium should or may be buffered depends on its intended purpose and is limited by the buffering capacity of the compounds used. Some large fermentation apparatuses are equipped with automatic controls that maintain a constant pH.

Miscellaneous physical requirements

Temperature, the gaseous environment, and pH are the major physical factors to be taken into consideration in establishing the optimum conditions for the growth of most species of bacteria. However, some bacteria have additional requirements. For example, phototrophic bacteria must be exposed to a source of illumination, since light is their source of energy. Bacterial growth may also be influenced by hydrostatic pressure. Bacteria have been isolated from the deepest ocean trenches where the pressure is measured in tons per square inch, and many of these organisms will not grow in the laboratory unless the medium is subjected to a similar pressure.

Experimental methods for studying micro-organisms

Experiment No 1 Date…….

Aim
To examine the source of contamination in the aseptic microbiological work

Principle
Microorganisms comprises of those organisms, which can not be seen through the naked eye. These are ubiquitous in nature i.e practically these are present everywhere in the environment e.g in soil, water, air, aerosol, body surfaces of human beings and animals and also in various extreme environments. These organisms contaminate all these sources and can create a great havoc in all microbiological works. Thus it is important to carry out all microbiological work under aseptic conditions and therefore it is necessary to examine the sources of the contamination and also check the sterility of the environment such as of that of growth room, laminar air flow benches, incubators etc. Therefore it is important to study following:

A. Transmission of microorganisms through air
B. Transmission of microorganisms through skin
C. Transmission of microorganisms through aerosol
D. Effect of light and height on contamination
E. Examine the sterility of laminar air flow bench and growth room

Requirements

Peptone, beef extract, NaOH, HCl, test tubes, beakers, pipette, measuring cylinder, cotton, pH paper, distilled water.

Equipment

Autoclave, burner, balance, laminar air flow station, etc.

Methods

First of all prepare Nutrient agar medium as per the requirement.
1. Preparation of Nutrient Agar medium:
2. Weigh 0.3 gm of beef extract, 0.5 gm of peptone and 1.5 gm of agar separately.
3. Dissolved beef extract and peptone in 100 ml of distilled water; for each set of experiment.
4. Noted the pH of the solution, and finally set it at 7.0 with the help of HCl/NaoH.
5. Then, added agar and mixed well.
6. Cotton plugged the media and is autoclaved at 121^0C for 15 minutes at 15 lbs/inch^2.
7. Poured the autoclaved medium into sterile autoclaved petriplates, and allowed them to solidify.

Experiment A: Transmission through Air
1. Marked the petriplates before using them.
2. Kept one petriplates as a "control" which is left unexposed.
3. Exposed a petriplate in the lab with fan and coolers off, for about 15 minutes.

4. Exposed another petriplates in a laminar air flow bench with UV light and fan off, for 15 minutes
5. Exposed another petriplate in a growth chamber
6. Exposed another petriplates in a growth room with AC "ON" for 15 minutes

Experiment B: Transmission through skin

1. A petriplate is marked and is divide into three equal zones with the help of a marker.
2. The three zones are labeled as zone 1, 2 and 3
3. Another petriplate is labeled as "Control" and is left blank unexposed.
4. In the zone 1, make an impression of unwashed thumb.
5. In the zone 2, make an impression of thumb washed with soap and drying in air.
6. In the Zone 3, make an impression of thumb washed with soap and swabbed with 70% recitified spirit and air dried.

Experiment C: Transmission through aerosol

1. A petriplate is labeled as "control" and is left unexposed.
2. Another petriplate is opened under the laminar air flow bench, and infront of it breathed heavily and labeled the plate as "aerosol-1".
3. Another plate is given the same treatment and is labeled as "aerosol-II".

Experiment D: To check the sterility of laminar air flow bench

1. Took one unexposed petriplate and labeled it as the "control".
2. Exposed a petriplate in a laminar air flow bench, with fans "ON" for 15 minutes.
3. Exposed a petriplate in a laminar air flow bench, with fans "OFF" for 15 minutes.
4. Exposed a petriplate in a laminar air flow bench, with UV "ON" for 15 minutes.
5. Exposed a petriplate in a laminar air flow bench, with UV "OFF" for 15 minutes.

Experiment E: To study the effect of light and height on contamination

1. Labeled an unexposed petriplate as "control".
2. Exposed two petriplate on the ground floor, one in light, and the other in shade.
3. Exposed another two petriplates on the top floor of the building one in light and other in shade.

Observation and result

Transmission through Air

Temperature of the Laboratory = ^{0}C
Temperature of the Growth room = ^{0}C
Temperature of the AC room = ^{0}C

S. No.	Places where Plates exposed	Bacteria	Fungi	Actinomycetes	Total Microbial Count / cfu
1.	Control				
2.	Exposed in laboratory with fans "OFF"				
3.	Exposed in laboratory with fans "ON"				
4.	Exposed in Growth room				
5.	Exposed in AC room				

Transmission through skin

S. No.	Places where Plates exposed	Bacteria	Fungi	Actinomycetes	Total Microbial Count
1.	Control				
2.	Unwashed Thumb pressed				
3.	Thumb washed with soap pressed				
4.	Thumb washed with soap and spirit pressed				

Transmission through aerosol

S. No.	Places where Plates exposed	Bacteria	Fungi	Actinomycetes	Total Microbial Count
1.	Control				
2.	Aerosol 1				
3.	Aerosol II				

To check the sterility of laminar air flow bench

S. No.	Places where Plates exposed	Bacteria	Fungi	Actinomycetes	Total Microbial Count
1.	Control				
2.	UV and Fan "OFF"				
3.	UV and Fan "ON"				
4.	UV "ON" and Fan "OFF				
5.	UV "OFF" and Fan "ON"				

To study the effect of light and height on contamination

S. No.	Places where Plates exposed	Bacteria	Fungi	Actinomycetes	Total Microbial Count
1.	Control				
2.	Ground floor / shade				
3.	Ground floor / light				
4.	Top floor / shade				
5.	Top floor / light				

Experiment No 2 Date…….

Aim

To perform the simple staining to compare morphology, shape and arrangements of bacterial cells

Principle

In simple staining, the bacterial smear is stained with a single reagent. Basic stains with a positively charged chromogen are preferred, since bacterial nucleic acids and certain cell-wall components carry a negative charge that strongly attracts and binds to chronic chromogen. The purpose of simple staining is to elucidate the morphology and arrangement of bacterial cells. The most commonly used basic stains are methylene blue, crystal violet and carbol fuchsin. It should be noted that exposure times differ for each other of these stains: carbol fuchsin requires 15-30 seconds, crystal violet 2-60 seconds and methylene blue 1-2 minutes.

Requirements

Cultures 24 hrs nutrient agar slants cultures of *Escherichia coli, Bacillus*

Reagents Methylene blue, crystal violet
Equipment Bunsen burner, inoculating loop, staining tray, microscope, lens paper, bibulous paper and glass slides.

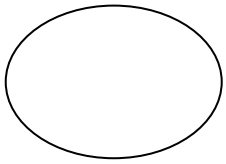
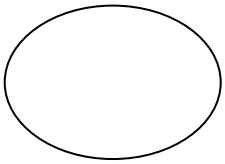
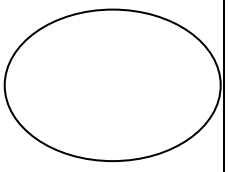
Methodology

1. Prepare separate bacterial smears of the organisms following the procedure described. Note: All smears must be heat fixed prior to staining.
2. Place a slide on the staining tray and flood the smear with one of the indicated stains, using the appropriate exposure time.
3. Wash smear with tap water to remove excess stain. During this step the slide should be held parallel to the stream of water, thereby reducing the loss of organims from the preparation.
4. Using bibulous paper, blot dry but do not wipe the slide.
5. This procedure is repeated with the remaining two prganisms, using a different stain for each.
6. Examine all stained slides under oil immersion.

Observation and Results

In the space provided:

1. Draw a representative field for each organism.
2. Described the morphology of the organism with reference to their shape (bacilli, cocci, spirilli) and arrangement (chains, clusters, pairs)

	Methylene blue	**Crystal violet**	**Carbol fuchsin**
Drawing of a representative field			
Organism	_____	_____	_____
Cell morphology			
Shape	_____	_____	_____
Arrangement	_____	_____	_____
Cell Color	_____	_____	_____

Experiment No 3 Date…….

Aim

Enumeration of bacterial population in soil by serial dilution-agar plating technique

Principle

Microorganisms are ubiquitous in nature and are found in soil, air, water, food, sewage and on body surface. Every area of our environment is replete with them. The ultimate sources of microorganisms for use in industrial processes are found in nature. Soil, living or decaying plant and animal matter, sewage sludge etc. provide a wide spectrum of microorganisms suited to many purposes with different functions and role. There are many techniques involved in the analysis of materials such as food, water, milk and soil for quantitative enumeration of microorganisms. In nature microbial populations do not segregate themselves by species but exist with a mixture of many other cell types. The microbiologist separates these mixed populations into individual species for study. Many methods have been devised to accomplish this, including direct microbial count, Breed smears, an electronic counter such as the coulter counter, chemical methods for estimating cell mass or cellular constituent, turibidimetric measurement for increase in cell mass and serial dilution agar plate technique.

Requirements

1. Sample for enumeration: Soil and waste water sample
2. Chemicals : Media

Nutrient agar	(gm/L)
Peptone	5
NaCl	5
Beef extract	1.5
Yeast extract	1.5
Agar	15
pH	7.4±2

   and 70% alcohol
3. Equipment: Laminar flow bench, incubator, autoclave, refrigerator
4. Glass Ware: Test tubes, test tube caps, petriplates, pipettes, spreader, inoculation needle, sterile sampling bottles, cotton, spatula, parafilm

Methodology

Sampling for bacteriological examination

The samples shall be representative of the water/soil to be tested, they should be collected with utmost care to ensure that no contamination occur at the time of collection or prior to the examination. Volume of the sample should be sufficient for carrying out all the tests required. The sampling bottles should not be filled up to the brim and 2-3 cm space should be left for effective shaking of the bottles.

All samples should be legibly marked with source of the sample, date and time of collection and the name and designation of the person collecting the sample.

Standard plate count method/spread-plate method

1. Prepare media by dissolving Nutrient agar in distilled water as per requirement.
2. Took 10 test tubes, each with nine ml of water.
3. Wrap pipettes in tin foil
4. Autoclave all above material at 120 ^{0}C for 20 minutes.
5. Then in laminar flow bench, add autoclaved material in petriplates.
6. Take one gram of soil and prepare a 10 fold dilution series upto 10^{-6}
7. Withdraw by sterile pipette 0.1 ml aliquots from dilution 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and dispense it in petriplates containing solidifying media. Spread this with spreader on the agar plate. Mark all the petriplates as per their dilution factor with marker.
8. Incubate the petriplates in incubator at temperature 30 ^{0}C for 24 hours
9. After a day calculate the no. of colonies in each petriplate.

Pour plate method

1. Prepare media by dissolving Nutrient agar in distilled water as per requirement
2. Transfer the desired amount of culture/inoculum/serial diluted suspension in the petriplae.
3. Dispense cooled (40-45^{O}C) agar media into the petriplate, mix well by rotating the petriplate and allow to solidify.
4. Seal the petriplae with a strip of parafilm and incubate in incubator for 24 hr.

Streak Plate method

1. Prepare nutrient agar media as described.
2. Autoclave all above material at 120 ^{0} C for 20 minutes at a pressure of 15 lbs /sq. in.
3. Then in laminar flow bench, add autoclaved media in petriplates. Mark the petriplate in four parts 1, 2, 3, 4, with marker after solidifying the media
4. Place a loop full of culture on agar surface in area 1. Flame and cool the loop, and drag it rapidly several times across the surface of area 1.
5. Reflame and cool the loop and turn the petridish 90 degrees. Then touch the loop to a corner of the culture in area 1 and drag it several times across the agar in area 2. The loop should never enter area 1 again.
6. Reflame and cool the loop and again turn the dish 90 degrees. Streak area 3 in the same manner as area 2.
7. Without reflaming the loop again, turn the dish and now drag the culture from the corner of area 3 across 4, using a wider streak. The loop must not touch any of the previously streaked areas.
8. Incubate the petriplates in incubator at 30^0 C for 24 hr.
9. After a day calculate the number of colonies in each petriplate.

Experiment No 4 Date…….

Aim

Enumeration of bacterial population in water and waste water by serial dilution-agar plating technique

Principle

Microorganisms are ubiquitous in nature and are found in soil, air, water, food, sewage and on body surface. Every area of our environment is replete with them. The ultimate sources of microorganisms for use in industrial processes are found in nature. Soil, living or decaying plant and animal matter, sewage sludge etc. provide a wide spectrum of microorganisms suited to many purposes with different functions and role. There are many techniques involved in the analysis of materials such as food, water, milk and soil for quantitative enumeration of microorganisms. In nature microbial populations do not segregate themselves by species but exist with a mixture of many other cell types. The microbiologist separates these mixed populations into individual species for study. Many methods have been devised to accomplish this, including direct microbial count, Breed smears, an electronic counter such as the coulter counter, chemical methods for estimating cell mass or cellular constituent, turibidimetric measurement for increase in cell mass and serial dilution agar plate technique.

Requirements

1. Sample for enumeration: Soil and waste water sample
2. Chemicals: Media

Nutrient agar	(gm/L)
Peptone	5
NaCl	5
Beef extract	1.5
Yeast extract	1.5
Agar	15
pH	7.4±2

   and 70% alcohol
3. Equipment: Laminar flow bench, incubator, autoclave, refrigerator
4. Glass Ware: Test tubes, test tube caps, petriplates, pipettes, spreader, inoculation needle, sterile sampling bottles, cotton, spatula, parafilm

Methodology

Sampling for bacteriological examination

The samples shall be representative of the water/soil to be tested, they should be collected with utmost care to ensure that no contamination occur at the time of collection or prior to the examination. Volume of the sample should be sufficient for carrying out all the tests required. The sampling bottles should not be filled up to the brim and 2-3 cm space should be left for effective shaking of the bottles.
All samples should be legibly marked with source of the sample, date and time of collection and the name and designation of the person collecting the sample.

Standard Plate Count method/ spread-plate method

1. Prepare media by dissolving Nutrient agar in distilled water as per requirement.
2. Took 10 test tubes, each with nine ml of water.
3. Wrap pipettes in tin foil
4. Autoclave all above material at 120 ^{0}C for 20 minutes.
5. Then in laminar flow bench, add autoclaved material in petriplates.
6. Take 1 ml of water sample and prepare a 10 fold dilution series upto 10^{-6}
7. Withdraw by sterile pipette 0.1 ml aliquots from dilution 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and dispense it in petriplates containing solidifying media. Spread this with spreader on the agar plate. Mark all the petriplates as per their dilution factor with marker.
8. Incubate the petriplates in incubator at temperature 30 ^{0}C for 24 hours
9. After a day calculate the no. of colonies in each petriplate.

Pour plate Method

1. Prepare media by dissolving Nutrient agar in distilled water as per requirement
2. Transfer the desired amount of culture/inoculum/serial diluted suspension in the petriplate.
3. Dispense cooled (40-45^O C) agar media into the petriplate, mix well by rotating the petriplate and allow to solidify.
4. Seal the petriplae with a strip of parafilm and incubate in incubator for 24 hr.

Streak Plate method

1. Prepare nutrient agar media as described.
2. Autoclave all above material at 120^0 C for 20 minutes at 15 lbs pressure.
3. Then in laminar flow bench, add autoclaved media in petriplates. Mark the petriplate in four parts 1, 2, 3, 4, with marker after solidifying the media
4. Place a loop full of culture on agar surface in area 1. Flame and cool the loop, and drag it rapidly several times across the surface of area 1.
5. Reflame and cool the loop and turn the petridish 90 degrees. Then touch the loop to a corner of the culture in area 1 and drag it several times across the agar in area 2. The loop should never enter area 1 again.
6. Reflame and cool the loop and again turn the dish 90 degrees. Streak area 3 in the same manner as area 2.
7. Without reflaming the loop again, turn the dish and now drag the culture from the corner of area 3 across 4, using a wider streak. The loop must not touch any of the previously streaked areas.
8. Incubate the petriplates in incubator at 30^0 C for 24 hr. After a day calculate the number of colonies in each petriplate.

Experiment No 5 Date…….

Aim

To study the bacterial cell morphology and determine the Gram character of bacteria by differential staining

Principle

Gram stain is the most important differential stain used in bacteriology and this special staining procedure was introduced in 1889 by a Danish physician named Christian Gram. It permits separation of bacteria into two kinds, the Gram positive organisms and the Gram negative species which makes it an essential tool for classification and differentiation of microorganisms. Differential staining requires the use of at least three chemical reagents that are applied sequentially to a heat fixed smear.

Requirements

Gram staining requires

1. Aqueous Crystal Violet: Acts as a primary stain to impart colour to all the cells.
2. Gram's Iodine: This reagent serves as a mordant and forms an insoluble complex by binding to the primary stain.
3. Decolorize: This acts as a lipid solvent and as a protein dehydrating agent and its action is determined by the lipid concentration of the microbial cell walls.
4. Counter Stain: Aqueous Safranin is the final reagent to stain red those cells that have not been previously decolourized.

1. Crystal violet (aqueous 1%)
   Crystal violet 1g
   Distilled water 100 ml
   Dissolve crystal violet in water, filter if necessary.
2. Gram's Iodine
   Potassium Iodide 2 g in 100 ml distilled water
3. Gram's decolouriser
   Acetone 50%
   Alcohol 50%
4. Safranin (aqueous 2%): 2 g in 100 ml of distilled water, mix well and filter if necessary.

Methodology

1. Preparation of a fixed bacterial smear: put a drop of bacterial culture on the slide with the help an inoculation needle and spread it in the center of the slide. Allow it to dry. Intermittent gentle heating is sometimes done to prepare heat fixed smear. If the bacterial growth is taken from slant then first put a drop of sterile saline (0.2%) distilled water on

the slide and suspend bacterial growth in it on the slide and spread using inoculation needle.
2. Place the slide on the staining rack and flood the smear with crystal violet for about 1 min.
3. Wash the stain gently with iodine solution. Stain with a fresh iodine solution for 1 min.
4. Wash in tap water or by dipping in a beaker containing water.
5. Add a few drops of decolorizer and continue until colour ceases to come out of the preparation. This may take 5 seconds to 1 minute.
6. Wash gently with water as in step 4.
7. Counter stain with dilute carbol fuchsin or safranin for 10-30 seconds.
8. Wash with water and dry off most of the slide with absorbent paper and leave the smear to dry by evaporation.
9. Dry slide is a permanent preparation and is examined under the microscope directly without a cover slip first under the low power and then under higher magnification. Examine under the oil immersion lens using cover slip.

Observation

Gram positive cells are purple and Gram negative cells are pink or red. Some species represent borderline cases and are best classified as Gram variable.

Microscopy and its different types

The basic microscopic system used in the microbiology laboratory is the light microscope. This instrument is also called as bright-field microscope because visible light passes directly through its lenses until it reaches the eye. Another common name is compound microscope because of its two-lens system, with the objective lens nearer the object and the ocular lens nearer the eye.

In light microscopy, visible light is projected through the opening in the focuses the light into a sharp cone. The light then passes through the opening in the stage, into the slide, and bounces off the object. Next, it enters the objective lens to form a magnified image darker than the background. This image is called a real image because it can be projected onto a screen. However, the image is not seen by the microscopist. Instead, the image becomes an object for the ocular lens, which magnifies the image a second time to create a virtual image in space. Only the observer can see this image. It appears about as distant from the eye as this page is from your end.

A light microscope usually has three objective lenses: the low-power, high-power and oil immersion lenses. In general, these lenses magnify an object 10, 40 and 100 times, respectively. The magnification is represented by the multiplication sign, x. The real image is then remagnified by the ocular lens. With a 10x ocular, the total magnification achieved are 10x, 400x, and 1000x respectively.

For an object to be seen distinctly the lens system must have good resolving power; that is, it must transmit light without variation and allow closely spaced objects to be clearly distinguished. For example, a car seen in the distance at night may appear to have a single headlight because the eyes lack resolving power. However, as the car comes closer, the two headlights can be seen

clearly as the resolving power of the eye increases. The headlights now have resolution or clarity.

The resolving power (RP) of a microscope is important because it denotes the size of the smallest object that can be seen clearly. Resolving powers vary for each objective lens and are calculated using the following formula:

$$RP = \frac{\lambda}{2 \times NA}$$

In this formula, the symbol λ (lambda) represents the wavelength of light and is usually set at 550 nm, the half way point between the limits of visible light. The symbol NA stands for the numerical aperture of the lens. This number is generally printed on the side of the objective lens. It refers to the size of the cone of light that enters the objective and the medium in which the lens is suspended, usually air. For a low-power objective with a NA of 0.25, the resolving power may be calculated as follows:

$$RP = \frac{550 \text{ nm}}{2 \times 0.25} = 550/0.5 = 1100 \text{ nm or } 1.1 \text{ μm}$$

Since the resolving power for this lens system is 1.1 μm, any object smaller than 1.1 μm could not be seen, but an object larger than 1.1 μm would be visible.

Another factor of the compound microscope is the working distance, the amount of clearance between the slide and the bottom of the objective lens. Working distance is related to where the object comes into focus. For the low-power objective, a common working distance is 6.8 millimeters (mm); for the oil-immersion objective, it is a scant 0.12 mm, almost 60 times closer.

When switching from the low power lens to the oil-immersion lens, one quickly finds that the image has become fuzzy. The object lacks resolution, and the resolving power of the lens system appears to be poor. This is because the objective lens should be used with immersion oil. The system's resolving power is calculated with the lens suspended in oil rather than air, a factor that increases the numerical aperture to 1.25.

Oil is needed for oil-immersion microscopy because light bends abruptly as it leaves the glass slide and enters the air. Both low-power and high-power objectives are wide enough to capture sufficient light for viewing, but the oil-immersion objective is so narrow that most light bends away and would miss the objective if oil were not used. The index of refraction (or refractive index) is a measure of the light-bending ability of a medium. Immersion oil has an index of refraction of 1.5, which is almost identical to the index of refraction of glass. Because the refractive index is the same for oil and glass, the light does not bend as it passes from the glass slide into the oil. By comparison, air has an index of refraction of 1.0, which accounts for the abrupt bending as light enters it. The oil thus provides a homogeneous pathway for light from the slide to the objective, and the resolution of the object increases.

Staining techniques

When preparing for light microscopy, microbiologists commonly stain bacteria because the cytoplasm of bacteria lacks color. Several techniques have been developed for this purpose.

To perform the simple stain technique, a small amount of bacterial suspension in water is placed on a glass slide and spread and the slide is air-dried. Next, the slide is passed through a flame in a process called heat fixing. This binds the cells to the slide, kills many organisms that may still be alive, and prepares them for staining. Now the slide is flooded with a basic dye such as crystal violet or methylene blue. Cytoplasm generally has a negative charge, and since basic dyes have a positive charge, the dye is attracted to the cytoplasm, where staining takes place.

The negative stain technique works in the opposite manner. Bacteria are mixed on a slide with an acidic dye such as nigrosin (a black stain) or Congo red (a red dye). The mixture is then smeared across the face of the slide and allowed to air-dry. Because the acidic dye carries a negative charge, it is repelled by the cytoplasm. The stain gathers around the negatively charged cells, and the microscopist observes clear or white cells on a colored background. Since this technique avoids chemical reactions and heat fixing, the cells appears less shriveled and less distorted and are closer to their natural condition.

The Gram stain technique allows us to view stained cells while learning something about them. The technique is named for Christian Gram, the Danish physician who first suggested its use in 1884. It is a differential technique because it differentiates bacteria into two groups depending on the results. Certain bacteria are called Gram positive bacteria; others are Gram-negative.

The first two steps of the technique are straightforward. Air-dried heat-fixed smears are stained with crystal violet, then with special Gram's iodine solution. All bacteria become blue-purple. Next the smear is rinsed with a decolorizer such as 95 percent alcohol or an alcohol-acetone mixture. At this point, certain bacteria lose their color and become transparent. These are the Gram-negative bacteria. Other bacteria retain the blue-purple stain. These are Gram-positive bacteria. When safranin, a red dye, is applied to the slide, only Gram negative organisms accept the stain. Thus at the technique's conclusion, Gram-positive bacteria are blue-purple while Gram-negative organisms appear orange or red. By observing the color of the cells at the conclusion of the process, one may decide the group of which the bacteria belong.

It is not totally clear why bacteria respond differently to the Gram stain technique. One theory suggests that crystal violet and iodine form a chemical complex in the bacterial cytoplasm. Since Gram-negative bacteria have lipid content in their cell walls, some microbiologists maintain that the alcohol dissolves the lipid and allows the crystal violet-iodine complex to leak out of the cytoplasm. Gram-positive theory points to the heavy concentration of peptidoglycan in the cell wall of Gram-positive bacteria. Peptidoglycan, a complex carbohydrate, is thought to trap the crystal violet-iodine complex in its many cross linkages. Gram-negative bacteria have considerably less peptidoglycan in their cells wall, hence they would trap less of the complex. Note that the words "positive" and "negative" are nothing more than convenient expressions and that electrical charge play a minimal role in Gram staining.

Knowing whether an organism is Gram-positive or Gram-negative is important for several reasons. For instance, microbiologists use results from the Gram stain technique to identify an unknown organism and classify it in Bergey's Manual. Gram-positive and Gram-negative bacteria differ in their susceptibility to chemical substances such as antibiotics (Gram-Positive bacteria are more susceptible to penicillin, Gram-negative to tetracycline), and they have different structural components (Gram-negative bacteria have more complex cell walls, with an outer membrane). They produce different types of toxic poisons as well.

Another differential staining technique is acid-fast technique which is used to identify members of genus *Mycobacterium,* one species of which causes tuberculosis. These bacteria are normally difficult to stain, but they stain red when treated with carbolfuchsin and heat (or lipid solubilizer). Then they retain their color when washed with a dilute acid-alcohol solution. Other bacteria lose their color easily during the acid-alcohol wash. The *Mycobacterium* species is therefore said to be acid-resistant or "acid-fast." (A blue counter-stain is used to give color to nonacid-fast bacteria). Because they stain red and break sharply when they reproduce, *Mycobacterium* species are euphemistically referred to as "red snappers".

Dark field and phase-contract microscopy

In dark-field microscopy, the background remains dark, and the object is illuminated. A special condenser mounted under the stage of the dark-field microscope scatters the light and causes it to hit the object from different angles. Some light bounces off the object into the lens to make the object visible, but the surrounding area appears dark because it lacks background light. The effect is similar to seeing the moon at night. In this case, sunlight from behind the Earth reflects off the moon and we can see it, but the sky appears dark because the sun is hidden.

Dark-field microscopy helps in the diagnosis of diseases caused by spiral bacteria because these organisms are near the limit of resolution and do not stain well. For example, *syphilis* is caused by *Treponema palladum,* a spiral bacterium with a diameter of about 0.15 μm. This bacterium may be observed in scrapings taken from a lesion of a person who has the disease.

Special microscope parts are also used in phase-contrast microscopy. A series of special condensers and filters split a light beam and throw the light rays slightly out of phase. The separated beams of light then pass through and around microscopic objects, and small differences in the densities of the objects show up as different degrees of brightness and contrast. With the phase-contrast microscope, microbiologists can see organisms alive and unstained. The fine structures of yeasts, molds and protozoa are studied with this instrument.

Fluorescence and Electron microscopy

Fluorescence microscopy has emerged to become a major asset to diagnostic and research laboratories. The technique has been applied for the identification of many microorganisms and is a mainstay of modern microbiology, especially in health-related issues. Microorganisms are coated with a fluorescent dye, such as fluorescein, then illuminated with ultraviolet light energy. The energy excites electrons in the dye, and they move to higher energy levels. However, the electrons quickly drop back to their original energy levels and give off the excess energy as visible light. The coated microorganisms appear to fluoresce.

An important application of fluorescence microscopy is in the fluorescent antibody technique used to identify an unknown organism. In one variation of this procedure, fluorescein is chemically united with antibodies, the protein molecules produced by the body's immune system when stimulated by a specific organism. "Tagged" antibodies result. Next, these antibodies are mixed with a sample of the unknown organism. If the antibodies are specific for that organism, they will bind to it and coat the cells with the dye. When subjected to ultraviolet light, the organisms will fluoresce. If the organisms fail to fluoresce, antibodies for a different organism are tried.

The electron microscope grew out of an engineering design made in 1933 by German physicist Ernst Ruska (winner of the 1986 Nobel Prize in Physics). Ruska showed that electrons flow in a sealed tube if a vacuum is maintained to prevent electron wandering. Magnets pinpoint the flow onto an object, where the electrons are absorbed or deflected, depending on the density of structures within the object. When projected onto a screen underneath, the electrons form an image that outlines the structures.

The key to electron microscopy is the extraordinarily short wavelength of the beam of electrons. Measured at 0.005 nm (compared to 550 nm for visible light), the short wavelength dramatically increases the resolving power of the system and makes possible the visualization of viruses, fine cellular structures, and large molecules such as DNA.

Two types of electron microscopes are currently in use. The first type, the transmission electron microscope (TEM), is used to photograph detailed structures within cells. Ultrathin sections of the object must be prepared because the electron beam can penetrate matter only a short distance. After embedding the specimen in a suitable mounting medium or freezing it, scientists cut the specimen into sections with a diamond knife. In this manner, a single bacterium can be sliced the long way into a hundred or more sections. It is also possible to fracture the cells after freezing with a special knife, and then view the cast rather than the cell itself. The cast allows the observation of surfaces outside the cell as well as within it. This technique is called freeze-fracturing.

Once sectioned, the portions of the object are stained with a heavy metal such as gold or palladium to make certain parts dense. Next, the microscopist inserts the sections into the vacuum chamber of the instrument and focuses a 10,000 volt electron beam on them using magnetic lenses. An image forms below. A photograph prepared from the image may be enlarged with enough resolution to achieve a total magnification of over 20 million times. Objects as small as 2.0 nm can be seen.

The second type is the scanning electron microscope (SEM). This instrument, developed in the late 1960s, enables researchers to see the surfaces of objects in the natural state and without sectioning. The specimens are placed in the vacuum chamber and covered with a thin coat of gold to increase electrical conductivity and decrease blurring. The electron beam then sweeps across the object and knocks loose showers of electrons, thereby producing a darker contrasting spot and a sense of three dimensions. Magnifications with the SEM are limited to about 75000 to 100,000 times, but the instruments is relatively easy to operate and gives vivid and undistorted views of an organism's surface details. The electron microscope has added immeasurably to our

understanding of the structure and function of microorganisms by letting us penetrate their innermost secrets.

Physical methods for the control of micro-organisms

Application of high temperatures for destruction of microorganisms

The killing action of heat is, as we have seen, a time temperature relationship affected by numerous conditions that must be taken into consideration in selecting the time and temperature required to reduce the microbial population to the desired level. Practical procedures by which heat is employed are conveniently divided into two categories: moist heat and dry heat.

Moist heat

The application of moist heat for inhibiting or destroying microorganisms is discussed by the method used to obtain the desired result.

Steam under pressure

Heat in the form of saturated steam under pressure is the most practical and dependable agent for sterilization. Steam under pressure provides temperatures above those obtainable by boiling. In addition, it has the advantages of rapid heating, penetration, and moisture in abundance, which facilitates the coagulation of proteins.

The laboratory apparatus designed to use steam under regulated pressure is called an autoclave. It is essentially a double jacketed steam chamber equipped with devices which permit the chamber to be filled with saturated steam and maintained at a designated temperature and pressure for any period of time. In the operation of an autoclave it is absolutely essential that the air in the chamber be completely replaced by saturated steam. If air is present, it will reduce the temperature obtained with the chamber substantially below that which would be realized if pure saturated steam were under the same pressure. It is not the pressure that kills the organisms but the temperature of the steam.

The autoclave is essential equipment in every microbiology laboratory. Many media, solutions, discarded cultures, and contaminated materials are routinely sterilized with this apparatus. Generally, but not always, the autoclave is operated at a pressure of approximately 15 lb/in^2 (at 121^oC). The time of operation to achieve sterility depends on the nature of the material being sterilized, the type of container, and the volume. For example, 1000 test tubes containing 10 ml each of a liquid medium can be sterilized in 10 to 15 min. at 121^oC; 10 litres of the same medium contained in a single container would require 1 h or more at the same temperature to ensure sterilization.

Fractional sterilization

Some microbiological media, solutions of chemicals, and biological materials cannot be heated above 100^oC without being damaged. If, however, they can withstand the temperature of free-flowing steam (10^oC), it is possible to sterilize them by fractional sterilization (tyndallization). This method involves heating the material at 100^oC on three successive days with incubation periods in between. Resistant spores germinate during the incubation periods; on subsequent

exposure to heat, the vegetative cells will be destroyed. If spores are present and do not germinate during the incubation periods, the materials will not be sterilized. An apparatus known as the Steam Arnold is used for this technique; however, it is also possible to operate an autoclave with free-flowing steam for this purpose.

Boiling Water

Contaminated materials or objects exposed to boiling water cannot be sterilized with certainty. It is true that all vegetative cells will be destroyed within minutes by exposure to boiling water, but some bacterial spores can withstand this condition for many hours. The practice of exposing instruments for short periods of time in boiling water is more likely to bring about disinfection (destruction of vegetative cells of disease-producing microorganisms) rather than sterilization. Boiling water can not be (and is not) used in the laboratory as a method of sterilization.

Pasteurization

Milk, cream, and certain alcoholic beverages (beer and wine) are subjected to a controlled heat treatment (called pasteurization) which kills microorganisms of certain types but does not destroy all organisms. Pasteurized milk is not sterile milk.

Dry heat

Hot air sterilization

Dry-heat or hot-air, sterilization is recommended where it is either undesirable or unlikely that steam under pressure will make direct and complete contact with the materials to be sterilized. This is true of certain items of laboratory glassware, such as petri dishes and pipettes, as well as oils, powders, and similar substances. The apparatus employed for this type of sterilization may be a special electric or gas oven or even the kitchen stove oven. For laboratory glassware, a 2-h exposure to a temperature of 160^{o}C is sufficient for sterilization.

Incineration

Destruction of microorganisms by burning is practical routinely in the laboratory when the transfer needle is introduced into the flame of the Bunsen burner. A note of caution should be added here. When the transfer needle is sterilized, care should be exercised to prevent spattering, since the droplets which fly off are likely to carry viable organisms. The danger from spattering can be greatly reduced or eliminated by using a Bunsen burner or an electric heat coil equipped with a tube into which the transfer needle can be inserted.

Incineration is used for the destruction of carcasses, infected laboratory animals, and other infected materials to be disposed of. Special precautions need to be taken to ensure that the exhaust fumes do not carry particulate matter containing viable microorganisms into the atmosphere.

Low Temperatures

Temperatures below the optimum for growth depress the rate of metabolism, and if the temperature is sufficiently low, growth and metabolism cease. Low temperatures are useful for preservation of cultures, since microorganisms have a unique capacity for surviving extreme cold. Agar-slant cultures of some bacteria, yeasts, and molds are customarily stored for long periods of time at refrigeration temperatures of about 4 to 7^{o}C. Many bacteria and viruses can be maintained in a deep freeze unit at temperatures from –20 to –70^{o}C. Liquid nitrogen, at a temperature of –196^{o}C, is used for preserving cultures of many viruses and microorganisms, as well as stocks of mammalian tissue cells used in animal virology and many other types of research. In all these procedures, the initial freezing kills a fraction of the population, but the survivors may remain viable for long periods.

From these facts it is immediately apparent that low temperatures, however extreme, cannot be depended upon for disinfection or sterilization. Microorganisms maintained at freezing or subfreezing temperatures may be considered dormant; they perform no detectable metabolic activity. This static condition is the basis of successful application of low temperatures for the preservation of foods. Thus from a practical stand point, high temperatures may be considered as microbiocidal and low temperatures (freezing or lower) as microbistatic.

Desiccation

Desiccation of the microbial cell causes a cessation of metabolic activity, followed by a decline in the total viable population. In general, the time of survival of microorganisms after desiccation varies, depending on the following factors.

1. The kind of microorganism
2. The material in or on which the organisms are dried
3. The completeness of the drying process
4. The physical conditions to which the dried organisms are exposed, e.g., light temperature and humidity.

Species of Gram-negative cocci such as gonococci and meningococi are very sensitive to desiccation; they die in a matter of hours. Streptococci are much more resistant; some survive weeks after being dried. The tubercle bacillus (*Mycobacterium tuberculosis*) dried in sputum remains viable for even longer periods of time. Dried spores of microorganisms are known to remain viable indefinitely.

In the process of lyophilization, organisms are subjected to extreme dehydration in the frozen state and then sealed in a vacuum. In this condition, desiccated (lyophilized) cultures of microorganisms remain viable for many years.

Osmotic pressure

When two solutions with differing concentrations of solute are separated by a semipermeable membrane, there will occur a passage of water, through the membrane, in the direction of the higher concentration. The trend is toward equalizing the concentration of solute on both sides of a membrane. The solute concentration within microbial cells is approximately 0.95 percent. Thus if cells are exposed to solutions with higher solute concentration, water will be drawn out of the

cell. The process is called plasmolysis. The reverse process, that is, passage of water from a low solute concentration into the cell, is termed plasmoptysis. The pressure built up within the cell as a result of this after intake is termed osmotic pressure. These phenomena can be observed more conveniently with animal cells since they do not have rigid cell walls. Plasmolysis results in dehydration of the cell, and as a consequence metabolic processes are retarded partially or completely. The antimicrobial effect is similar to that caused by desiccation. Because of the great rigidity of microbial cell walls (except for protozoa), the cell-wall structure does not exhibit distortions as a result of plasmolysis or plasmoptysis. However, changes in the cytoplasmic membrane, and particularly shrinkage of the protoplast from the cell wall, can be observed during plasmolysis.

Radiation

Energy transmitted through space in a variety of forms is generally called radiation. For our purposes, the most significant type of radiation is probably electromagnetic radiation, of which light and X-rays are examples. Electromagnetic radiation has the dual properties of a continuous wave phenomenon and a discontinuous particle phenomenon; the particles are packets, or quanta, of energy, sometimes called photons, which vibrate at different frequencies. Radiation of a given frequency can also be described by its wavelength,λ: it is measured in angstroms, where 10,000 Å = 1 μm, and the energy of the radiation in electron volts (ev) is given by $12{,}350/\lambda$.

Electromagnetic radiation can interact with matter in one of two general ways. Gamma rays and x-rays, which have energies of more than about 10 eV, are called ionizing radiations because they have enough energy to knock electrons away from molecules and ionize them. When such radiations pass through cells, they create free hydrogen radicals, hydroxyl radicals, and some peroxides which in turn can cause different kinds of intracellular damage. Moreover, since this damage is produced in a variety of materials, ionizing radiations are rather nonspecific in their effects. Less energetic radiation, particularly ultraviolet light, does not ionize, it is absorbed quite specifically by different compounds because it excites electrons and raises them to higher energy levels, thus creating different chemical species that can engage in a variety of chemical reactions not possible for unexcited molecules.

In addition to electromagnetic radiation, organisms may be subjected to acoustic radiation (sound waves) and to subatomic particles, such as those released in radioactive decay. The atomic era has alerted us to the damaging potential of radiation. Consequently, a tremendous expenditure of research effort is being directed toward determining the minimum dosage which affects cells, how radiations damage cells, and how the damage can be prevented. Microorganisms have been used for the major part of this research for the same reasons they are used in so many other areas of basic biological research; they are easy to grow and lend themselves to rapid, efficient experimentation.

Besides the fundamental research in radiation microbiology, there have been many developments in the application of ionizing radiation to sterilize biological materials. This method is called cold sterilization because ionizing radiations produce relatively little heat in the material being irradiated. Thus it is possible to sterilize heat-sensitive substances, and such techniques are being developed in the food and pharmaceutical industries.

Ultraviolet Light

The ultraviolet portion of the spectrum includes all radiations from 150 to 3900 Å have the highest bactericidal efficiency. Although the radiant energy of sunlight is partly posed of ultraviolet light, most of the shorter wavelengths of this type are filtered out by the earth's atmosphere (ozone, clouds and smoke). Consequently, the ultraviolet radiation at the surface of the earth is restricted to the span from about 2670 to 3900 Å. From this we may conclude that sunlight, under certain conditions, has microbicidal capacity, but to a limited degree.

Many lamps are available which emit a high concentration of ultraviolet light in the most effective region, 2600 to 2700 Å. Germicidal lamps, which emit ultraviolet radiations, are widely used to reduce microbial populations. For example, they are extensively used in hospital operating rooms, in aseptic filling rooms, in the pharmaceutical industry, where sterile products are being dispensed into vials or ampoules, and in the food and dairy industries for treatment of contaminated surfaces.

An important practical consideration in using this means of destroying microorganisms is that ultraviolet light has very little ability to penetrate matter. Even a thin layer of glass filters cut off a large percentage of the light. Thus only the microorganisms on the surface of an object where they are exposed directly to the ultraviolet light are susceptible to destruction.

Mode of action

Ultraviolet light is absorbed by many cellular materials but most significantly by the nucleic acids, where it does the most damage. The absorption and subsequent reactions are predominantly in the pyrimidines of the nucleic acid. One important alteration is the formation of a pyrimidine dimmer in which two adjacent pyrimidines become bonded. Unless dimers are removed by the specific intracellular enzymes, DNA replication can be inhibited resulting in mutations.

X-rays (Roentgen rays)

X-rays are lethal to microorganisms and higher forms of life. Unlike ultraviolet radiations, they have considerable energy and penetration ability. However, they are impractical for purposes of controlling microbial populations because (1) they are very expensive to produce in quantity and (2) they are difficult to utilize efficiently, since radiations are given off in all directions from their point of origin. However, X-rays have been widely employed experimentally to produce microbial mutants.

Gamma Rays

Gamma radiations are high-energy radiations emitted from certain radioactive isotopes such as ^{60}Co. As a result of the major research programs with atomic energy, large quantities of radioisotopes have become available as by-products of atomic fission. These isotopes are potential sources of gamma radiations. Gamma rays are similar to X-rays but are of shorter wavelength and higher energy. They are capable of great penetration into matter, and they are lethal for all life, including microorganisms.

Because of their great penetrating power and their microbicidal effect, gamma rays are attractive for use in commercial sterilization of materials of considerable thickness or volume, e.g. packaged foods and medical devices. However, certain technical problems must be resolved for practical applications, e.g. development of radiation sources for large-scale use and design of equipment to eliminate any possible hazards to the operators.

Results of quantitative studies on the effect of ionizing radiations on the cells have resulted in the establishment of the "target" theory of action. This implies that the radiant –energy particle makes a "direct hit" on some essential substance such as DNA within the bacterial cell, causing ionization which results in the death of the cell.

Cathode rays (Electron-beam radiation)

When a high-voltage potential is established between a cathode and an anode in an evacuated tube, the cathode emits beams of electrons, called cathode rays or electron beams. Special types of equipment have been designed which produce electrons of very high intensities (millions of volts), and these electrons are accelerated to extremely high velocities. These intense beams of accelerated electrons are microbicidal as well as having other effects on biological and nonbiological materials.

The electron accelerator, a type of equipment which produces the high-voltage electron beam, is used today for the sterilization of surgical supplies, drugs and other materials. One of the unique features of the process is that the material can be sterilized after it has been packaged (the radiations penetrate the wrappings) and at room temperature. Electron-beam radiation has limited power of penetration; but within its limits of penetration, sterilization is accomplished on very brief exposure.

Surface tension and interfacial tension

The interface, or boundary, between a liquid and a gas is characterized by unbalanced forces of attraction between the molecules in the surface of the liquid and in the interior. A molecule at the surface of the liquid-air interface is pulled strongly toward the interior of the liquid beneath it, whereas molecules in the interior of the liquid are attracted uniformly in all directions. This behavior of the molecular forces at the liquid-air interface imparts a distinctive characteristic to the surface of a liquid, known as surface tension. Surface force also exists between two immiscible liquids and at the interface between a solid and liquid. Here they are referred to as interfacial tension. Changes in surface tension may alter the permeability characteristics of the cytoplasmic membrane, causing leakage of cellular substances, which results in damage to the cell.

Filtration

For many years a variety of filters have been available to the microbiologist which can remove microorganisms from liquids or gases. These filters are made of different materials – an asbestos pad in the Seitz filter, diatomaceous earth in the Berkefeld filter, porcelain in the Chamberland-Pasteur filter, and sintered glass disks in other filters.

The mean pore diameter in these bacteriological filters ranges from approximately one to several micrometers, most filters are available in several grades based on the average size of the pores. However, it should be understood that these filters do not act as mere mechanical sieves; porosity alone is not the only factor preventing the passage of organisms. Other factors, such as the electric charge of the filter, the electric charge carried by the organisms, and the nature of fluid being filtered, can influence the efficiency of filtration.

A new type of filter termed as the membrane of molecular filter has been developed whose pores are of a uniform and specific predetermined size. Membrane or molecular filters are composed of biologically inert cellulose esters. They are prepared as circular membranes of about 150 μm thickness and contain millions of microscopic pores of very uniform diameter. Filters of this type can be produced with known porosities ranging from approximately 0.01 to 10 μm. Membrane filters are used extensively in the laboratory and in industry to sterilize and fluid materials. They have been adapted to microbiological procedures for the identification and enumeration of microorganisms from water samples and other materials.

It is customary to force the fluid through the filter by applying a negative pressure to the filter flask by use of a vacuum or water pump or to impose a positive pressure above the fluid in the filter chamber, thus forcing it through. Upon completion of filtration, precautions must be taken to prevent contamination of the filtered material when it is transferred to other containers.

The development of high-efficiency particulate air (HEPA) filters has made it possible to deliver clean air to an enclosure such as a cubicle or a room. This type of air filtration together with a system of laminar airflow is also used to produce dust and bacteria free air. A summary of the application of physical agents for the control of microorganisms is provided in Table 1.

Table 1: Physical agents for controlling microorganisms

Method	Recommended Limitations	Limitations
Moist heat		
Autoclave	Sterilizing instruments, linens, utensils and treatment trays, media and other liquids	Ineffective against organisms in materials impervious to steam; cannot be used for heat-sensitive articles
Free-flowing steam or Boiling water	Destruction of non-sporeforming pathogens, sanitizes bedding, clothing and dishes	Cannot be guaranteed to produce sterilization on one exposure
Dry heat		
Hot Air Oven	Sterilizing materials impermeable to or damaged by moisture, e.g. oils, glass, sharp instruments, metals	Destructive to materials which cannot withstand high temperatures for long periods.
Incineration	Disposal of contaminated objects that cannot be reused.	Size of incinerator must be adequate of to burn largest load promptly and completely; potential of air pollution

Radiation		
Ultraviolet light	Control of airbone infection, disinfection of surfaces	Must be absorbed to be effective (does not pass through transparent glass or opaque objects); irritating to eyes and skin; low penetration
X-ray, gamma, and cathode radiation	Sterilization of heat sensitive surgical materials and other medical devices	Expensive and requires special facilities for use
Filtration		
Membrane filters	Sterilization of heat-sensitive biological fluids	Fluid must be relatively free of suspended particulate matter
Fiberglass filters (HEPA)	Air disinfection	Expensive
Physical cleaning		
Ultrasonics	Effective in decontaminating delicate cleaning instruments	Not effective along, but as adjunct procedure enhances effectiveness of other methods.
Washing	Hands, skin, objects	Sanitizes: reduces microbial flora

Methods of quantification of bacteria

The purpose of this section is to review the methods available for quantification of microorganisms in the environment. Quantification of bacterial cells is fundamental to most of the microbiological studies, therefore, rapid and simple techniques are required. The methods can be classified into two major types, i.e. culture dependent and culture independent methods depending upon whether the bacteria are culturable or not. In culture dependent methods only culturable bacteria can be quantified while the detection and quantification of viable but non-culturable bacteria (VBNC) cannot be done as these methods do not allow the detection of VBNC. For bacterial enumeration mostly culture dependent methods are used, however, for correct enumeration results of bacterial numbers including starved, injured and VBNC, it is important to use culture-independent methods (McFeters, 1990).

Culture dependent methods

Culture dependent methods represent the earliest techniques used to detect the microorganisms in the environment. Due to their simplicity and low cost, culture dependent methods are widely used for bacterial quantification. The main limitation of the method is that only culturable microorganisms can be quantified and in the environment only <10% of all microorganisms are reported to be culturable. Hence, the methods cannot quantify all microorganisms present in the environment. Following are the culture dependent methods that are used for bacterial quantification:

1. *Bacterial enumeration*

   This method is dependent upon the suspension-dilution of sample and inoculation on growth media with an appropriate dilution. After incubation, the microorganisms can be

quantified by counting the number of colonies present on the solid media plate. Counting is done when the microorganisms are present as discrete colonies and each colony represent one microorganism. The results are expressed as colony forming unit per gram weight of sample (CFU/g of sample). The advantage of the method is that it is easy to conduct, less expensive and very sensitive upto 102 to 103 CFU/g and the disadvantage is that it is time consuming and labor intensive. Also, sometimes it underestimates the CFU/g when some of the bacteria remain adhered to soil particles or get killed during the serial dilution and sometimes due to aggregation one colony may originate due to more than one microorganism.

2. *Most Probable Number (MPN) method*

It is the method used to quantify the number of microorganisms in aqueous samples without direct counting (Makkar and Casida, 1987; Jones and Knowles, 1991). It is proposed to be more reliable than growth on selective media for enumeration of microorganisms for epidemiological studies because of the significance of the lower detection level. The random sampling evaluation of ground beef from retail stores indicated that 39% of the samples were Listeria spp.-positive and 31% were L. monocytogenes-positive when using the colony count method. A total of 56% of the meat samples were found to be Listeria spp.-positive and 38% L. monocytogenes-positive when the MPN-method was used. Serial dilution of the sample estimates the densities of microorganism present on the basis that one microorganism will produce a positive result after incubation. It requires the use of probability tables to process the data that contribute in the reduction of sensitivity of analysis as compared to that of plating or enumeration.

3. *Molecular marker based method*

These methods combine aspects of colorimetric based and molecular based methods for the detection and quantification of microorganism present in the environment. The marker gene can be used to distinguish a particular microorganism containing a known gene from the backgrounds microbial population which do not possess the gene and that particular microbial population can be quantified by enumeration on selective media containing substrate. The marker genes can be of following types:

a) Use of chromogenic markers

Chromogenic markers contain one or more genes whose presence in a microbial cell may be detected by an ability to produce a color change in a substrate. Isolation of microorganisms on media supplemented with substrates that are capable of being transformed into colored products has been successfully used to distinguish bacteria expressing xylE gene, encoding for 2,3-dioxygenase. Other systems in this category are lacZY genes encoding for β-galactosidase (lacZ) and lactose permease (lacY), gusA gene encoding for β-glucuronidase etc. The coloured colonies can be quantified by plate counting and enumeration method, luminometry or by imaging. The disadvantage of the system is that the marked cells must be metabolically active.

b) Use of bioluminescent marker genes

Bioluminescent marker genes encode bioluminescent or fluorescent products, e.g., lux operon from Vibrio fischeri, in which organisms are identified by their ability to bio-luminesce, gfp (green fluorescent protein) from Aequorea victoria and luc from Photinus sp. The bioluminescent colonies can be counted directy by plate counting or by color photography or by direct microscopy with CCD (Charge Couple Device) camera. The disadvantage of the system is that the substrates used are too expensive and work at a particular pH, e.g., substrate D-luciferin is expensive and is only freely permeable below pH 5.0, thereby restricting its use to acidic environments only.

c) Antibiotic resistance marker gene*s*

These marker genes show resistance towards particular antibiotics, e.g., genes conferring resistance towards Kanamycin Rifampicin, Streptomycin etc. During the 1980's antibiotic resistance genes were introduced as markers for selection. As a result of the presence of the antibiotic resistance gene, few cells that contain the introduced DNA were able to grow and multiply in the presence of the antibiotic unlike the surrounding non-transformed cells that either die or have severely inhibited growth. The colonies that appear on the selective media containing antibiotics can be quantified by plate counting. The disadvantages of the method is that transformed cells convert the antibiotic to a detoxified compound that may still have negative effect on cell proliferation and differentiation and the public perception of the risk of antibiotic resistance.

Quantification of non-culturable microorganisms

Culture independent methods

The advantage of culture independent methods is that for the quantification of microorganisms in the environment, the microorganisms do not need to be extracted from the environment prior to detection. Therefore, these methods are able to quantify non-culturable bacteria also. These methods may be expected to quantify a more representative proportion of the microorganisms present in an environment. Following are the culture independent methods of microbial quantification:

1. Microscope based technique

This method is commonly used to enumerate microorganisms in environmental samples, without prior culturing, it is also referred as direct-counting. Direct cell counts, as determined by epifluorescence microscopy measured more microorganisms as compared to viable counts on selective media, indicating that the major portion of the microorganisms in the soil environment are VBNC. The number of viable or active cells can also be determined with activity stains, e.g., 2-(4-iodophenyl)-3-(4-nitrophenyl)-5 phenyltetrazolium.

2. Enzyme Linked Immunosorbant assay (ELISA) method

It can be used quantitatively to detect the presence of microorganisms in the environmental sample. ELISA combine the specificity of antibodies with the sensitivity of simple enzyme

assays, by using antibodies or antigens coupled to an easily-assayed enzyme. ELISAs can provide a useful measurement of antigen or antibody concentration. There are two main variations on this method: The ELISA can be used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen. The main disadvantage with the method is that it is not a straightforward method to be performed also there are chances of cross reactivity and the detection limit of 105 organisms per ml of sample is not sensitive.

3. Flow cytometry

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. Flow cytometry employs instrumentation that scans single cells flowing past excitation sources in a liquid medium. The technology can provide rapid, quantitative, multiparameter analyses on single living (or dead) cells based on the measurement of visible and fluorescent light emission. Flow cytometry is a widely used method for characterizing and separating individual cells. This basic protocol focuses on the measurement of fluorescence intensity produced by fluorescent-labeled antibodies and ligands that bind specific cell-associated molecules. Flow cytometry offers several advantages over conventional methods. In the flow cytometer measurements are made on individual cells and so sample heterogeneity can be quantified. This means that under some circumstances it may be possible to identify subpopulations of cells or to detect contaminants. Flow cytometry is a rapid technique; measurements are typically made at rates of 1000 cells s-1. This means that many thousands of cells can be measured in a realistic time scale.

The method detects all particles based on their light scattering properties. There is no requirement that the cells will grow on a designated medium or even that they be alive. This total count can be combined with a viability stain where a measure of the viable microbial load is required. In addition, a suitable fluorescent stain may be added to the sample prior to analysis enabling simultaneous determination of both total and viable counts.

3. Molecular methods

Nucleic acid based methods involve the detection and identification of microorganisms on the basis of their genetic material, i.e., DNA or RNA. The advantage of this method is that the microorganisms need not to be cultured for their analysis and it is applicable to greater proportion of microbial community. The heterogeneity of extracted DNA is a measure of total number of genetically different bacteria in environment, which in turn provides a picture of the dynamics of total numbers of microbial cells. Following are the culture independent molecular methods for the quantification of microorganisms:

Polymeras Chain Reaction (PCR)

PCR is applicable to the study of microorganisms in a range of environmental samples. It is a method for amplifying a sequence of DNA using heat stable polymerase and primers complementary to the two strands of DNA. Amplification of DNA in a sample by PCR is required if the microorganisms are present in numbers of <106 ml-1. PCR has been the most

sensitive method for detection of specific DNA in environmental samples, sensitivities of 1-100 cells per gram of soil has been reported. PCR is used to amplify a short, well-defined part of a DNA strand. This can be a single gene, or just a part of a gene. As opposed to living organisms, the PCR process can copy only short DNA fragments, usually up to 10 kb. Certain methods can copy fragments up to 40 kb in size, which is still much less than the chromosomal DNA of a eukaryotic cell, for example, a human cell contains about three billion base pairs. There are some partial modifications to the PCR which are as follows:

i) Nested PCR

It is intended to reduce the contaminations in products due to the amplification of unexpected primer binding sites. Two sets of primers are used in two successive PCR runs, the second set intended to amplify a secondary target within the first run product. This is very successful, but requires more detailed knowledge of the sequences involved.

ii) Inverse PCR

It is a method used to allow PCR when only one internal sequence is known. This is especially useful in identifying flanking sequences to various genomic inserts. This involves a series of digestions and self ligation before cutting by an endonuclease, resulting in known sequences at either end of the unknown sequence.

iii) RT-PCR

Reverse Transcription PCR is the method used to amplify, isolate or identify a known sequence from a cell or tissues RNA library. Essentially normal PCR preceded by transcription by Reverse transcriptase (to convert the RNA to cDNA) this is widely used in expression mapping, determining when and where certain genes are expressed.

iv) Asymmetric PCR

Asymetric PCR is used to preferentially amplify one strand of the original DNA more than the other. It finds use in some types of sequencing and hybridization probing where having only one of the two complementary stands is ideal. PCR is carried out as usual, but with a great excess of the primers for the chosen strand. Due to the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required. A recent modification on this process, known as Linear-After-The-Exponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperture (Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

v) Quantitative PCR

Quantitative PCR is used to rapidly measure the quantity of PCR product (preferably real-time), thus is an indirect method for quantitatively measuring starting amounts of DNA, cDNA or RNA. This is commonly used for the purpose of determening whether a sequence is present or not, and if it is present the number of copies in the sample. There are 3 main methods which vary in difficulty and detail.

vi) Quantitative real-time PCR

It is often confusingly known as RT-PCR (Real Time PCR). QRT-PCR or RTQ-PCR are more appropriate contractions. RT-PCR can also refer to reverse transcription PCR, which even more confusingly, is often used in conjunction with Q-PCR. This method uses fluorescent dyes and probes to measure the amount of amplified product in real time.

Preservation and maintenance of microbial cultures

Slants and Broths

Use of slants and broth cultures for maintaining primary stock cultures is probably the simplest method available. For the majority of microorganisms, mature cultures grown on slants or in broth may be refrigerated and thereby preserved for some time, usually for a few months. However, use of slants and broths presents some serious shortcomings and, in general, it is the least desirable method available. It necessitates the frequent transfer of cultures which greatly increases the chance of contamination and undesirable genetic change. Many fungi, for example, grown under the artificial conditions of slants lose their ability to perform a desired biochemical process after several transfers. Bacteria, like fungi may lose some of the desired properties such as virulence, antigenicity, or the ability to produce a particular metabolite.

Some of the problems just mentioned may be minimized by altering the media in which the micro organisms are subcultured. This helps to prevent the natural selection for strains which grow well in a particular medium. Much attention has been given to the composition of media which may be used successfully to culture microorganisms but the selection must be tailored to each particular situation.

Oil Overlay

Mineral oil may be used to overlay the mature growth of microorganisms on a slant. This process reduces water loss by evaporation, slows the exchange of gas, and allows subculturing without destruction of the primary stock culture. But use of the oil overlay method is subject to objections similar to those for the broth or slant method, such as loss of sporulation, loss of biochemical activity etc. The technique is, however, particularly useful in maintaining fungi which do not produce spores.

Soil

A third and relatively simple method of preserving cultures is the dry spore stock on sterile soil. This method has been used successfully for a variety of microorganisms including fungi and bacteria. The first consideration in using soil should be to note whether soil is to be used strictly as a carrier or also as a growth medium. If used as a carrier, abundant spores of the bacterial or fungal species must be prepared in advance. The mature spores may then be placed in sterile soil and the resulting preparation may be dried in air or under vacuum.

For many fungi, maintenance by use of soils stocks is very useful. Moist soil may be inoculated and fungus may be allowed to grow until sporulation has been completed. This type of culture may be used both as primary stock and as a working stock culture.

Freezing

When the need for long time storage arises, it is necessary to reduce the metabolic activity of microbial cells to such a point that reproduction is halted. Presently two methods are employed to reach this goal. The biochemical activity of cells can be reduced to a state of "suspended animation" by reducing their temperature by the almost complete removal of water.

Freezing cells is a harsh process which can damage cells beyond repair. Methods have been developed which allow freezing with minimum damage to assure the recovery of live cells of many microorganisms. A considerable amount of information has been published on the events that take place during the freezing process and many opinions have been advanced on the best method to be used. The following area a few generally accepted rules on the frozen storage of live cells.

1. The temperature drop should be about 1oC per min to –20oC; then as rapid as possible to the storage temperature.
2. Thawing should be as rapid as possible.
3. Survival rates may be increased by the addition to glycerol, sugars, or other protective agents.
4. Electrolytes should be kept at a minimum.

Liquid Nitrogen

In recent years the use of liquid nitrogen for ultra low temperature storage cells, including microbial cells, has become a very useful method for long term preservation. Assuming that cellular metabolism is completely stopped at temperatures of –130oC, organisms should be able to survive for an indefinite period of time provided they can withstand the cooling and thawing process. This process has been used successfully for a wide variety of cells including fungi, bacteriophages, protozoa, algae, mammalian cells, and bacteria.

The overall process for ultralow temperature preservation is similar to that for the freezing process described earlier. A cell suspension protected by such materials as glycerol is cooled at a rate of about 1oC per min until a temperature of –35oC is reached. At this point the temperature is allowed to drop at an uncontrolled rate.

Some differences of opinion exist as to the method by which the frozen cells should be thawed. Several studies have conducted. Generally the tubes are removed directly from the low temperature storage and placed in a 37oC – 40oC water both and agitated for rapid thawing. Caution should be exercised during the thawing process. A cracked or otherwise faulty ampoule may have become contaminated with liquid nitrogen. As thawing proceeds the pressure created by passage of this liquid nitrogen to the gas phase may cause an explosion.

The use of liquid nitrogen storage of microbial cells has several advantages: No subculturing of cells; non-spore forming microbes such as basidiomycetes may be preserved; no changes in the biochemical mechanisms or the genetic equipment of the cells; no contamination problems once the ampoules are stored. The disadvantages are: The initial cost of the equipment; the need for a constant supply of liquid nitrogen; and the difficulty in distributing cultures to other laboratories.

Lyophilization

From the author's point of view, lyophilization is probably the most satisfactory method for long term preservation of those organisms which will withstand the process. For this reason lyophilization will be described in more detail than the previous methods.

Although it had been used earlier, the lyophilization process was developed into a valuable tool during the 1940s primarily by the Northern Regional Laboratory in Peoria, III. Later work at this institute turned this process into a very useful and highly reliable method for the preservation of many types of microorganisms (Fennel 1960; Collins and Lyne 1976). It requires no subculturing of cells; there is no change in the biochemical reactions of the cells, and the cells are genetically stable; there are no contamination problems once the ampoules have been sealed; the finished ampoules can be easily shipped by mail; and the initial costs are much less than those for liquid nitrogen storage. The major disadvantage of the lyophilization process is simply that not all microorganisms can be preserved by this method.

Lyophilization or freeze drying consists primarily of suspending propagative cells (bacteria or yeast cells, conidia, ascospores, etc.) in a protective medium, freezing and the removal of water by sublimation under reduced pressured. The desiccated cultures are then sealed under vacuum and stored at low temperature (4oC). The majority of molds (pencillia, spergilli, Mucorales) survive well, whereas Pythicacea, Entomophtorales, large spored fungi, and mycelial forms seldom survive the initial treatment. Many other single celled organisms such as yeast and bacteria also can be preserved by this method. If the organisms survive the initial treatment, they are likely to remain viable for a period of 20 years or more.

The spores or cells may be suspended in various protective media such as bovine serum or media containing sugars. However, skim milk is probably the most desirable medium, and it is more easily obtained. The microbial suspension is dispensed into small ampoules using sterile techniques. The ampoules are then quick frozen at about –35oC and placed under a vacuum of at least 200 μm of Hg. After drying, the ampoules are sealed under vacuum.

Ampoules for lyophilization may be prepared by cutting pyrex glass tubing into lengths of approximately 11 cm. One end of the tube is then sealed by rapidly rotating it in an oxygen-natural gas flame. Care must be used to seal the end completely. The other end is then fire polished to smooth the rough edges. Before lyophilization the tubes should be snugly stoppered with cotton and sterilized. If the cotton stoppers are too tight, the frozen cell suspension will melt during the process resulting in an unusable preparation.

A suspension medium prepared from a mixture of 1 part fresh skim milk and 1 part distilled water should be sterilized. A cell suspension is prepared by introducing the sterile skim milk water mixture into a fresh fungus culture about 2 days after sporulation has been completed. A

highly concentrated suspension of fungi spores increases the chance of recovery of the culture after lyophilization. Approximately 3 drops of the cell suspension should be placed into each ampoule with a sterile Pasteur pipette. The excess cotton should be cut from the stopper leaving a stopper about 8-10 mm long which is pushed to within 10 mm of the medium. A small tag showing the culture identification on one side and the date on the other wide is also placed inside the ampoule.

The preparation may be frozen in a beaker of ethanol with chips of dry ice at a temperature of –35oC to – 40oC. It is important that the temperature should not rise above –35oC during freezing. Temperatures below –35oC are usually not damaging to the culture, while temperature above –35oC may result in poor viability.

Once the preparations are frozen the tubes should be subjected to the vacuum. A vacuum of 200 μm Hg is usually satisfactory. The actual vacuum is not really critical. The important point is that the frozen preparation not melts during the process. Often it is necessary to maintain the frozen preparation in an ice bath for the first few minutes of drying. After the cultures are dry, the tubes are sealed under vacuum with an oxygen gas torch. Finished cultures may be stored at room temperature. However, it is advisable to store the ampoules in the dark since light can reduce viability and storage in the refrigerator is recommended.

Several methods may be used to open the tubes, but sterility must be maintained. Thump the tube with the fingers to break up the pellet. Wipe the surface of the tube with a sterilizing solution (7% ethanol or Chlorox 1:1) after making a file scratch across the center of the cotton plug. Apply a red hot glass to the scratch to crack the glass or simply apply pressure to break glass tubing. Use care in opening the ampoules as the contents are under vacuum. Allow time for air, filtered by the plug, to seep into the ampoule. Otherwise, when the pointed end is snapped off the plug will be drawn to one end. Hasty opening may release live particles of the dried organism into the air of the laboratory. Since the cotton plug and the tube may contain spores or cells they should be autoclaved after use. If the tube does not have a cotton stopper make a file scratch on the sealed tube, sterile the outer surface with ethanol or some other disinfectant, and break open inside a wrapping of sterile cotton.

Reculturing

One of the several methods may be used for reculturing. Regardless of the method, the resulting culture should be compared with a description of the culture originally lyophilized.

Method A

After opening the tubes, introduce a small volume of sterile water equal in volume to the pellet, and replace the cotton plug. Allow the ampoule to stand for 30 min, and then streak the suspension on agar medium. For bacteria, blood agar should be used since some bacteria require haemin for recultivation.

Method B

Prepare 50 ml of a suitable culture medium, place in a 250 ml Erlenmeyer flask, stopper and sterilize. The contents of the ampoule may then be dumped into the flask. This method does not allow for the possibility that contamination may have occurred (as does the streaking method).

Patience must be used in reculturing. Often several days to a week are required for growth to appear.

A medium suitable for the growth of each individual microorganism should be used for reculturing lyophilized cultures. Some investigations have shown that malt extract increases the chance of satisfactory results in reculturing fungi, and malt extract is generally included in reculturing media.

The main cause of failure to recover lyophilized cultures is poorly sealed tubes which lose their vacuum. A few days after lyophilization, the vacuum in the tubes should be checked. This can be done by using an induction coil (spark coil tester, high frequency coil, or generator, Tesla type). The tubes may be lined up side by side on a table top at room temperature. Touching the end of the tube with the discharge tip of the tester results in a white to purplish glow in the tube. Absence of this glow indicates a loss of vacuum.

Many factors influence the survival rates of microorganisms preserved by lyophilization. These have previously been reviewed. The most important generalization about lyophilization is simply this; if the culture withstands the original process of lyophilization, it is likely to survive for many years as long as the culture remains under vacuum.

Importance of pure culture methods

The food fermentations which are the subject of subsequent chapters are not pure culture fermentations. In some instances, they still depend on the mixed microflora of the raw material (e.g. sauerkraut or some sourdough fermentations). However, the major food fermentations depend on massive inoculation with microbial "seed" derived from a pure strain. The use of commercial bakers' yeast in doughs or the use of the bacterial starter cultures in the production of cheese is characteristic of modern food fermentations. This massive inoculation is based on the selection of an appropriate strain and on its maintenance as a pure strain. This is true whether the microbial seed is commercially available (e.g. bakers' yeast) or whether it is produced "in house" (e.g. brewers' yeast). Therefore, the subject matter of the present chapter, "obtaining pure cultures" and "maintaining pure cultures" is fundamental to the commercial practice of food fermentations.

Suggested Reading

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