BACTERIOLOGY PART 3

NUTRITIONAL REQUIREMENTS OF BACTERIA

Nutrition is the part of microbial physiology that deals with the nutrients required for growth. Different organisms need different quantities of nutrients, and not all nutrients are required in the same amounts. Some nutrients, called **macronutrients or macroelements**, are required in large amounts, while others called **micronutrients or trace elements**, are required in just trace amounts.

1. **Macronutrients or Macroelements**

Chemical analysis of cells shows that over 95% of cell dry weight is made up of a few major elements: carbon, oxygen, hydrogen, nitrogen, sulphur, phosphorus, potassium, calcium, magnesium, and iron. These are the macronutrients or macroelements. The first six elements namely carbon, oxygen, hydrogen, nitrogen, sulphur, phosphorus, dominate in all living systems and are essential being components of carbohydrates, lipids, proteins, and nucleic acids. They are namely carbon (C), oxygen (O), hydrogen (H), nitrogen (N), sulphur (S), and phosphorus (P).

The remaining four macronutrients or macroelements exist in the cell as cations and play a variety of roles. For example, potassium (K^+) is required for activity by a number of enzymes, including some involved in protein synthesis. Calcium (Ca^{2+}), among other functions, contributes to the heat resistance of bacterial endospores. Magnesium (Mg^{2+}) serves as a cofactor for many enzymes, complexes with ATP, and stabilizes ribosomes and cell membranes. Iron (Fe^{2+} and Fe^{3+}) plays a major role in cellular respiration. Iron is a key component of cytochromes and of iron–sulphur proteins involved in electron transport reactions leading to the synthesis of ATP.

(a) Requirements for Carbon, Hydrogen, Oxygen and Electrons

All organisms need carbon, hydrogen, oxygen, and a source of electrons. Carbon is needed to synthesize the organic molecules from which organisms are built. Hydrogen and oxygen are also important elements found in many organic molecules. Electrons are needed for two reasons: (a) for provision of energy used in cellular work via the movement of electrons through electron transport chains and during other oxidation-reduction reactions (b) to reduce molecules during biosynthesis (e.g., the reduction of CO_2 to form organic molecules). The requirements for carbon, hydrogen, and oxygen usually are satisfied together because molecules serving as carbon sources often contribute hydrogen and oxygen as well. For instance, many **heterotrophs** —organisms that use reduced, preformed organic molecules as their carbon source—can also obtain hydrogen, oxygen, and electrons from the same molecules. Because the

electrons provided by these organic carbon sources can be used in electron transport as well as in other oxidation-reduction reactions, many heterotrophs also use their carbon source as an energy source. Indeed, the more reduced the organic carbon source (i.e., the more electrons it carries), the higher its energy content. Thus, lipids have a higher energy content than carbohydrates.

Other microbes are **autotrophs** —organisms that use carbon dioxide (CO_2) as their sole or principal source of carbon. Although CO_2 is plentiful, its use as a carbon source presents a problem to autotrophs. CO_2 is the most oxidized form of carbon, lacks hydrogen, and is unable to donate electrons during oxidation-reduction reactions. Therefore, CO_2 cannot be used as a source of hydrogen, electrons, or energy. Because CO_2 cannot supply their energy needs, autotrophs must obtain energy from other sources, such as light or reduced inorganic molecules.

(b) Requirements for Nitrogen, Phosphorus, and Sulphur

To grow and reproduce, a microorganism must be able to incorporate large quantities of nitrogen, phosphorus, and sulphur. Although these elements may be acquired from the same nutrients that supply carbon, microorganisms usually employ inorganic sources as well.

The bulk of nitrogen available in nature is in inorganic form as ammonia (NH_3), nitrate (NO_3^-), or nitrogen gas (N_2). Nitrogen is needed for the synthesis of amino acids, purines, pyrimidines, some carbohydrates and lipids, enzyme cofactors, and other substances. Many microorganisms can use the nitrogen in amino acids. Others can incorporate ammonia directly through the action of enzymes such as glutamate dehydrogenase or glutamine synthetase and glutamate synthase. Virtually all prokaryotes can use NH_3 as their nitrogen source, and many can also use NO_3^-. By contrast, N_2 can only be used by nitrogen-fixing prokaryotes e.g., *Rhizobium*.

Phosphorus is present in nucleic acids, phospholipids, nucleotides such as ATP, several cofactors, some proteins, and other cell components. Almost all microorganisms use inorganic phosphate (PO_4^{2-}) as their phosphorus source and incorporate it directly.

Sulphur is present in the amino acids cysteine and methionine and also in several vitamins, including thiamine, biotin, and lipoic acid. Sulphur is equally needed for the synthesis of substances such as the amino acids cysteine and methionine, some carbohydrates, biotin, and thiamine. Most microorganisms use sulphate (SO_4^{2-}) as a source of sulphur after reducing it; a few microorganisms require a prereduced form of sulphur such as cysteine. Others use it in form of sulfide (HS^-).

2. **Micronutrients or trace elements**

These are several nutrients needed by all microorganisms in small amounts —amounts so small that in the lab they are often obtained as contaminants in water, glassware, and growth media. Likewise in nature, they are ubiquitous and usually present in adequate amounts to support the growth of microbes. The micronutrients—manganese, zinc, cobalt, molybdenum, nickel, and copper—are needed by most cells. Micronutrients are normally a part of enzymes and cofactors, and they aid in the catalysis of reactions and maintenance of protein structure. For example, zinc (Zn^{2+}) is present at the active site of some enzymes but can also be involved in the association of different subunits of a multimeric protein. Manganese (Mn^{2+}) aids many enzymes that catalyze the transfer of phosphate. Molybdenum (Mo^{2+}) is required for nitrogen fixation, and cobalt (Co^{2+}) is a component of vitamin B_{12}.

3. **Growth Factors**

Growth factors are organic compounds that, like trace metals, are required in only very small amounts. Growth factors are vitamins, amino acids, purines, pyrimidines, or various other organic molecules. Although most microorganisms are able to biosynthesize the growth factors they need, some must obtain one or more of them from the environment and thus must be supplied with these compounds when cultured in the laboratory because they lack the enzymes or biochemical pathways needed for their synthesis.

There are three major classes of growth factors: (1) amino acids, (2) purines and pyrimidines, and (3) vitamins. Amino acids are needed for protein synthesis, purines and pyrimidines for nucleic acid synthesis. **Vitamins** are small organic molecules that usually make up all or part of enzyme cofactors. They are needed in only very small amounts to sustain growth.

Some microorganisms are able to synthesize large quantities of vitamins needed by humans. These microbes can be used to manufacture these vitamins for human use. Several water-soluble and fat-soluble vitamins are produced partly or completely using industrial fermentations. Examples of such vitamins and the microorganisms that synthesize them are riboflavin (*Clostridium, Candida*), coenzyme A (*Brevibacterium*), vitamin B_{12} (*Streptomyces, Propionibacterium, Pseudomonas*), vitamin C (*Gluconobacter, Erwinia, Corynebacterium*), β-carotene (*Dunaliella*), and vitamin D (*Saccharomyces*).

CULTIVATION AND GROWTH OF MICROORGANISMS

Microbial growth is the orderly increase in cellular constituents that ultimately results in the formation of new cells. Growth in most bacteria is exhibited or shown by transverse binary fission to produce two daughter cells of

equal size and composition. Nuclear division is followed by cell division. Before dividing, a cell must reproduce all of its essential constituents and double its mass. It is usually not convenient to investigate the growth and reproduction of individual microorganisms because of their small size. Therefore, when studying growth, microbiologists normally follow changes in the total population number. That is why for the microbiologists, growth is defined as an increase in the number of cells.

Microbial cells have a finite life span, and a species is maintained only as a result of continued growth of its population. There are many reasons why it is important to understand how microbial cells grow. For example, many practical situations call for the control of microbial growth, in particular, bacterial growth. Secondly, knowledge of how microbial populations can rapidly expand is useful for designing methods to control microbial growth, whether the methods are used to treat a life-threatening infectious disease or simply to disinfect a surface. Thirdly, knowledge of the events surrounding bacterial growth also allows us to see how these processes are related to cell division in higher organisms because there are many parallels.

Bacterial Generation Time or Cell Cycle

The bacterial generation time or cell cycle is the complete sequence of events extending from the formation of a new cell through the next cell division. It is equally called the population doubling time. In other words, it is the interval of time between two cell divisions giving rise to two daughter cells under optimum conditions. In all cases, when one cell eventually separates to form two cells, we say that one generation has occurred. During one generation, all cellular constituents increase proportionally; cells are thus said to be in balanced growth. The time required for a generation in a given bacterial species is highly variable and is dependent on nutritional and genetic factors, and temperature. Under the best nutritional conditions, the generation time of a laboratory culture of *E. coli* and many other medically important bacteria, is about 20 min. Some bacteria are slow-growing. The generation time in tubercle bacilli is about 20 hours. In lepra bacilli, it is as long as about 20 days.

The Purpose of Cultivation and Identification of Microorganisms

Cultivation is defined as the process of propagating or producing organisms by allowing them to reproduce in predetermined culture media under a well-controlled laboratory conditions of time, temperature, and atmospheric conditions.

In natural habitat, microorganisms usually grow in complex, mixed population with many species. This presents a problem for microbiologists, technologists, and technicians who work in clinical microbiology and research

laboratories because a single type of microorganism cannot be studied adequately in a mixed culture. One needs a pure culture of the organism to study and identify. A pure (or axenic) culture is a population of cells growing in the absence of other species or types. A pure culture may originate from a single cell or single organism, in which case the cells are genetic clones of one another and thus are genetically identical.

Culture techniques are used in medical diagnostic laboratories for the following purposes:

(a) To isolate pathogens in pure cultures.

(b) To identify the pure cultures of the isolated pathogens and

(c) To test for their sensitivity (susceptibility) to antimicrobials if their presence is indicated.

In microbiology research laboratories microorganisms are cultured or cultivated for the following reasons:

(a) To study and learn more about them

(b) To harvest antibiotics and other microbial products

(c) To test new antimicrobial agents and

(d) To produce vaccines

Factors which Affect Bacterial Growth

Bacterial growth is affected by many different environmental factors, including the availability of nutrients and moisture, osmotic pressure, temperature, pH, and aeration. These environmental factors affect microorganisms in our daily lives and play important roles in the control of microorganisms in laboratory, industrial, and hospital settings. Whether scientists wish to encourage or inhibit the growth of microorganisms, they must first understand the fundamental needs of microbes.

1. Availability of Nutrients

All living organisms require nutrients—the various chemical compounds that organisms use to sustain life. Therefore, to survive in a particular environment, appropriate nutrients must be available. Many nutrients are energy sources; organisms will obtain energy from these chemicals by breaking chemical bonds. Nutrients also serve as sources of carbon, oxygen, hydrogen, nitrogen, phosphorus, and sulfur as well as other elements (e.g., sodium, potassium, chlorine, magnesium, calcium, and trace elements such as iron, iodine, zinc, etc.) that are usually required in lesser or trace amounts.

The nutritional requirements of each microorganism depend on that organism’s metabolic capabilities. A cell that is unable to synthesize an essential nutrient must be able to acquire that nutrient from its growth medium. For many organisms, a single compound (such as amino acid) may serve as

energy source, carbon source and nitrogen source; others require a separate compound for each.

Bacteria can be classified nutritionally based on their energy requirements and on their ability to synthesize essential metabolites. Bacteria which derive energy from sunlight are called **phototrophs**. The process by which organisms convert light energy into chemical energy is called photosynthesis. Those that obtain energy from chemical reactions are called **chemotrophs**. Bacteria that can synthesize all their organic compounds are called **autotrophs**. They are able to use atmospheric carbon dioxide and nitrogen. They are capable of independent existence in water and soil. They are of no medical importance. Some bacteria are unable to synthesize their own metabolites. They depend on preformed organic compounds. They are called **heterotrophs**. These bacteria are unable to grow with carbon dioxide as the sole source of carbon. Their nutritional requirements vary widely.

2. Water availability (Moisture) and Osmotic Pressure (Ionic Srength)

All organisms need water to grow, but not necessarily to survive – many, particularly those forming spores, can survive well without water. Hence, they grow well only when adequate moisture is present. The amount of 'free' water available for growth in a culture medium (water that is not bound by hydrogen bonding) is indicated by the Water Activity (symbol Aw, measured on a scale from 0 to 1, where 1 represents pure water with no dissolved solutes). Bacteria normally require higher water activity values than fungi. Keeping materials free of moisture by dehydration is one of the most common methods of controlling growth of microorganisms. Frequently, dehydrated foods such as powered milk contain large number of viable organisms. A lack of moisture, however, maintains the microorganisms in a static state so that multiplication cannot occur.

Water availability not only depends on the absolute water content of an environment, that is, how moist or dry it is, but it is also a function of the concentration of solutes such as salts, sugars, or other substances that are dissolved in the water. Dissolved substances have an affinity for water, which makes the water associated with solutes less available to organisms. Hence the osmotic pressure of a solution will rise as the amount of solute is increased so, generally, solutions containing large amounts of dissolved sugars or salts will have low water activities. Many organisms grow best at osmotic pressures similar to those in their 'natural' environment.

Water diffuses from regions of high water concentration (low solute concentration i.e., low osmotic pressure) to regions of lower water concentration (higher solute concentration i.e., high osmotic pressure) in the

process of osmosis. The cytoplasm of a cell typically has a higher solute concentration (higher osmotic pressure) than the environment, so water tends to diffuse into the cell and cause it to burst unless something is done to prevent the influx or inhibit plasma membrane expansion. However, if the cell brings under control the influx or inhibits plasma membrane expansion, the cell is said to be in positive water balance. However, when a cell finds itself in an environment where the solute concentration exceeds that of the cytoplasm, water will flow out of the cell. This can cause serious problems if a cell has no way to counteract it because a dehydrated cell cannot grow. Therefore, microorganisms can be affected by changes in the osmotic concentration of their surroundings. If a microorganism is placed in a hypotonic solution (one with a lower osmotic concentration), water will enter the cell and cause it to burst unless something is done to prevent the influx or inhibit plasma membrane expansion. Conversely if it is placed in a hypertonic solution (one with a higher osmotic concentration), water will flow out of the cell. In microbes that have cell walls, the membrane shrinks away from the cell wall—a process called plasmolysis. Dehydration of the cell in hypertonic environments may damage the cell membrane and cause the cell to become metabolically inactive. This is the basis of using high concentrations of salt or other solutes in preserving foods against microbial attack.

The rigid cell walls of bacteria together with their inclusion bodies and other mechanisms help them to survive in hypotonic environments. Besides these mechanisms, many microorganisms, whether in hypotonic or hypertonic environments, keep the osmotic concentration of their cytoplasm somewhat above that of the habitat by the use of compatible solutes, so that the plasma membrane is always pressed firmly against their cell wall. **Compatible solutes** are solutes that do not interfere with metabolism and growth when at high intracellular concentrations. Compatible solutes are used inside the cell for adjustment of cytoplasmic water activity and they must be noninhibitory to macromolecules within the cell.

Organisms able to live in environments high in sugar as a solute are called **osmophiles**, and **Halophiles**, (salt loving) e.g., the archaeon *Halobacterium* and marine organisms require high concentration of salts (usually above 12 % Na salts) for growth. Halophiles grow in the Great Salt Lake in Utah and in food products preserved with salt. **Halotolerant** microorganisms are those organisms that do not require NaCl for growth but can grow in the presence of NaCl, in some cases, substantial levels of NaCl e.g., *Staph. aureus* which is selectively isolated in a medium containing about 7.5 % salt. Those organisms which are able to grow in very dry environments (made dry by lack of water rather than from dissolved solutes) are called **xerophiles** e.g., *Halobacterium*.

3. Temperature

Temperature is probably the most important environmental factor affecting the growth and survival of microorganisms. At either too cold or too hot a temperature, microorganisms will not be able to grow and may even die. Bacteria vary in their requirement of temperature for growth. Hence, bacteria growth rates are greatly affected by temperatures.

Temperature affects microorganisms in two opposing ways. As temperatures rise, chemical and enzymatic reactions in the cell proceed at more rapid rates and growth becomes faster; however, above a certain temperature, cell components may be irreversibly damaged due to instability of its membranes, and proteins. Thus, as the temperature is increased within a given range, growth and metabolic function increase up to a point where the high temperature denatures enzymes, transport carriers, and other proteins. At high temperatures, the lipid bilayer simply melts and disintegrates. Above this point, cell functions fall to zero. For every microorganism, there is a minimum temperature below which growth is not possible because membranes gel or solidify, an optimum temperature at which growth is most rapid and maximal, and a maximum temperature above which growth is not possible. The growth temperature optimum reflects a state in which all or most cellular components are functioning at their maximum rate and is typically closer to the maximum than to the minimum. These three temperatures, called the **cardinal temperatures**, are characteristic for any given microorganism. Thus, when organisms are above their optimum temperature, both function and cell structure are affected. If temperatures are very low, function is affected but not necessarily cell chemical composition and structure. Beyond their effect on growth rate, extremes of temperature kill microorganisms. Hence, extreme heat is used to sterilize preparations. Extreme cold also kills microbial cells, although it cannot be used safely for sterilization.

Microorganisms are organized into five groups according to the temperature range in which they grow namely:

(a) Psychrophiles

Psychrophiles grow well at 0°C and have an optimum growth temperature of 10°C or lower; the maximum is around 15°C. They are readily isolated from Arctic and Antarctic habitats. Oceans constitute an enormous habitat for psychrophiles because 90% of ocean water is 5°C or colder. Examples include members of the genera *Pseudomonas*, *Flavobacterium* and *Alcaligenes*. Psychrophiles are found in unusually cold environments.

(b) Psychrotrophs (facultative psychrophiles)

These organisms grow at 0 to 7°C even though they have optima between 20 and 30°C, and maxima at about 35°C. Psychrotrophic bacteria and fungi are major causes of refrigerated food spoilage.

(c) Mesophiles

These are microorganisms with growth optima around 20 to 45°C. They often have a temperature minimum of 15 to 20°C, and their maximum is about 45°C or lower. Most microorganisms probably fall within this category. Almost all human pathogens are mesophiles, as might be expected because the human body is a fairly constant 37°C. Mesophiles are widespread in nature. They are found in warm blooded animals and in terrestrial and aquatic environments in temperate and tropical latitudes. *Escherichia coli* is a typical mesophile.

(d) Thermophiles

Thermophiles grow at temperatures between 55 and 85°C. Their growth minimum is usually around 45°C, and they often have optima between 55 and 65°C. Thermophiles are found in unusually hot environments. These organisms flourish in many habitats including composts, self-heating hay stacks, hot water lines, and hot springs. Representative member is *Geobacillus stearothermophilus*.

(e) Hyperthermophiles

Hyperthermophiles have growth optima between 85°C and about 113°C. They usually do not grow well below 55°C. Hyperthermophiles are found in extremely hot habitats such as hot springs, geysers, and deep-sea hydrothermal vents. *Pyrococcus abyssi* and *Pyrodictium occultum* are examples of marine hyperthermophiles found in hot areas of the seafloor.

4. Acidity and Alkalinity (pH)

The relative acidity or alkalinity of a solution is expressed by its pH on a scale on which neutrality is pH 7. pH values less than 7 are acidic and those greater than 7 are alkaline. The acidity or alkalinity of an aqueous environment has a major effect on and determines the organisms that can live in it. Each species has a definite pH growth range and pH growth optimum. Based on pH microorganisms can be classified either as **neutrophiles**, **acidophiles** or **alkalophiles**. **Acidophiles** have their growth optimum between pH 0 and 5.5; **neutrophiles,** between pH 5.5 and 8.0; and **alkalophiles (alkaliphiles),** between pH 8.0 and 11.5. Extreme alkalophiles have growth optima at pH 10 or

higher. Though extremes of pH exist, the pH of most natural environments is close to neutrality, which is why most microbes are neutrophiles.
In general, different microbial groups have characteristic pH preferences. Most bacteria and protists are neutrophiles. Most fungi prefer more acidic surroundings, about pH 4 to 6; photosynthetic protists also seem to favor slight acidity. Many archaea are acidophiles. For example, the archaeon *Sulfolobus acidocaldarius* is a common inhabitant of acidic hot springs; it grows well from pH 1 to 3 and at high temperatures. The archaea *Ferroplasma acidarmanus* and *Picrophilus oshimae* can actually grow very close to pH 0.

Unlike in most natural environments where the pH is not significantly affected by the metabolism of microorganisms, the pH of laboratory **batch cultures** is subject to changes resulting from metabolic activities of the microbes that consume or produce acidic or basic substances. For example, many fermenting bacteria produce organic acids, including lactic acid, formic acid, acetic acid, succinic acid and propionic acid and fermentation of amino acids releases ammonia, which raises the pH. When too much acid or alkali accumulates, there is a decrease in microbial growth. Thus, buffers are usually included in some culture media to keep the pH relatively constant and prevent extreme changes in pH. Many media are buffered by potassium phosphate which also functions as the cell's source of phosphate and potassium.

5. Availability of Oxygen

Because animals require molecular oxygen (O_2), it is easy to assume that all organisms require oxygen. However, this is not true; many microorganisms can, and some must, live in the total absence of oxygen. Microorganisms vary in their need for, or tolerance of, oxygen as a means of producing energy. We have two major groups namely **aerobes** and **anaerobes**. Aerobes are able to grow in the presence of atmospheric oxygen whereas anaerobes can grow in the absence of atmospheric oxygen.

Aerobes are further divided into three subgroups: **obligate aerobes**, **facultative anaerobes (or facultative aerobes)**, and **microaerophiles**. Obligate aerobes grow in the presence of full atmospheric oxygen tension (21 % oxygen). They are completely dependent on oxygen for growth. However, facultative anaerobes (or facultative aerobes) do not require oxygen for growth but grow better in its presence. In the presence of oxygen, they use oxygen as the terminal electron acceptor during aerobic respiration. Most pathogenic microorganisms are either obligate aerobes or facultative anaerobes; obligate anaerobic pathogens are less common. Microaerophiles such as *Campylobacter*, *Helicobacter*, and *Streptococcus* are damaged by the normal atmospheric level of oxygen (21%) and require oxygen levels in the range of 2 to 10% for growth.

Anaerobes on the other hand have the following subgroups namely: **obligate anaerobes**, and **aerotolerant anaerobes.** Strict or obligate anaerobes are usually inhibited or killed in the presence of oxygen (e.g., *Bacteroides,Clostridium, Methanococcus*). Aerotolerant anaerobes can tolerate oxygen and grow in its presence even though they cannot use it. They simply ignore it and grow equally well whether it is present or not e.g., *Enterococcus faecalis*.

The toxicity of 0_2 results from its reduction by enzymes in the cell (such as flavoproteins) to hydroxyl radical (OH•), and hydrogen peroxide (H_20_2) and by ferrous ion to the even more toxic free radical, superoxide (0_2^-).

$O_2 + e - \rightarrow O_2^-$ (superoxide radical)

$O_2^- + e - + 2H^+ \rightarrow H_2O_2$ (hydrogen peroxide)

$H_2O_2 + e - + H^+ \rightarrow H_2O + OH\bullet$ (hydroxyl radical)

These products oxidize and rapidly destroys cellular constituents. Neutrophils and macrophages use these products of oxygen reduction to kill pathogens. Obligate aerobes, facultative anaerobes and aerotolerant anaerobes are shielded from the toxicity of oxygen in the following ways: Obligate aerobes, and facultative anaerobes usually contain the enzymes usually contain the enzymes **superoxide dismutase (SOD)** and **catalase (CAT),** which catalyze the destruction of superoxide radical and hydrogen peroxide, respectively as shown in the equation below.

$2O_2^- + 2H^+ \xrightarrow{SOD} 0_2 + H_20_2$

$2H_20_2 \xrightarrow{CAT} 2H_20 + 0_2$

$H_2O_2 + NADH + H^+ \rightarrow 2H_2O + NAD^+$ ... Catalylsed by Peroxidases

Aerotolerant microorganisms (e.g., *Enterococcus faecalis* a lactic acid bacteria) do not contain catalase but rely instead on **peroxidases**, which reduce hydrogen peroxide to water at the expense of oxidizable organic substrates as expressed in the third equation above. However, they usually have **superoxide dismutase**. All strict anaerobes lack both enzymes or have them in very low concentrations and therefore cannot tolerate oxygen. However, some microaerophilic bacteria and anaerobic archaea protect themselves from the toxic effects of oxygen with the enzymes **superoxide reductase** and peroxidase. Superoxide reductase reduces superoxide to H_2O_2 without producing oxygen. The H_2O_2 is then converted to water by **peroxidase**.

In the laboratory, growth of aerobic bacteria is achieved by supply of air to cultures through either mechanically shaking the culture vessel to introduce

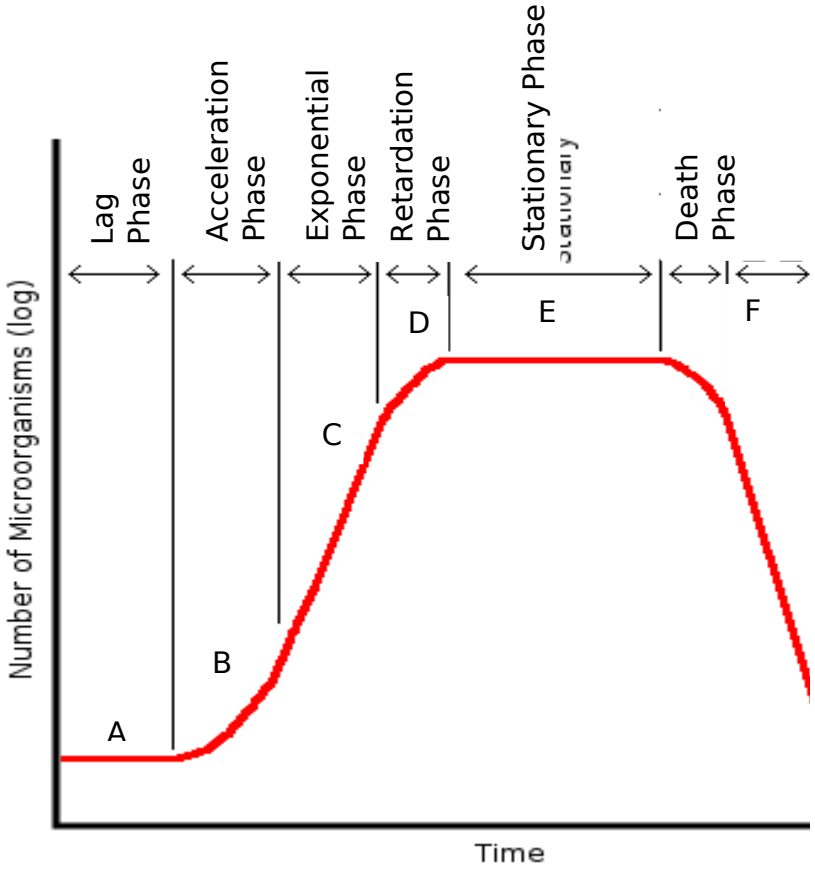
oxygen into the medium, or forcing air through the medium by pressure or by simply growing the microbes on agar slants, e.g., stock cultures of aerobes are often maintained on agar slants to provide a large surface area exposed to air. The diffusion of oxygen often becomes the limiting factor in growing aerobic bacteria.

Laboratory culturing of anaerobes, on the other hand require the exclusion of oxygen from the medium. This could be achieved by addition of reducing agents such as sodium thioglycolate to liquid cultures, sealing of tubes of agar with a layer of petrolatum and paraffin, placing the culture vessel in a container from which oxygen has been removed either chemically or by evacuation or by handling the organism within an anaerobic jar.

Bacterial Population Growth Curve

Experiments in microbial growth are usually done on cell populations because it is too difficult to study individual cells. A population growth curve for any particular species of bacterium may be determined by growing a pure culture of the organism in a liquid medium at a constant temperature. Such a liquid medium is called batch culture because it is a closed system i.e., a closed culture vessel with a single batch of medium which is not replenished nor waste removed. Because no fresh medium is provided during incubation, nutrient concentrations decline and concentrations of wastes increase as the bacteria grow to saturation.

When bacterial cells from such closed system are transferred to a fresh liquid medium, if the number of viable cells per milliliter is determined periodically and then plotted, a curve of the type shown below is obtained and represents a typical bacterial growth curve for a liquid culture. The curve may be discussed in terms of 6 phases, represented by the letters A – F.



Bacterial Growth Curve

Summary of Phases of the bacterial growth curve;

Section of Curve	Phase	Growth Rate
A	Lag	Zero
B	Acceleration	Increasing
C	Exponential	Constant
D	Retardation	Decreasing
E	Stationary	Zero
F	Decline /Death	Negative

The Lag Phase

When a microbial culture is inoculated into a fresh medium, growth usually begins only after a period of time called the lag phase. During this period, cells transferred to a new medium adjust to the new environment. The cells are metabolically active and may increase in size, but they do not divide.

During the lag phase, the transferred cells synthesize the enzymes and metabolites they need to grow at a maximal rate in the new environment. At the same time the toxic products from the previous growth medium are either metabolized by the cells or diluted by the new medium. Clinically, lag phase is the period during which pathogenic organisms adapts to the host environment.

The duration of the lag phase may be brief or extended depending on the physiological condition of the transferred cells and the growth phase. If an exponentially growing culture is transferred into the same medium under the same conditions of growth (temp., aeration, and the like), there is no lag and exponential growth begins immediately. However, if the inoculum is taken from an old (stationary phase) culture and transferred into the same medium, there is usually a lag, even if all the cells in the inoculum are alive. This is because the cells are depleted of various essential constituents and time is required for their biosynthesis. Hence the length of the lag phase will also depend on the length of time the culture was held in the stationary phase. A lag occurs also when the inoculum consists of cells that have been damaged (but not killed) by significant temperature shifts, radiation, or toxic chemicals because of the time required for the cells to repair the damage. A lag is also observed when a microbial population is transferred from a rich culture to a poorer one; for example, from a complex medium to a defined medium. The lag phase ends when bacterial growth rate begins to increase (acceleration phase).

The Acceleration Phase

This represents the period when the bacterial cells, having synthesized the enzymes and metabolites required for growth starts to multiply. There is increasing growth rate as more cells multiply until a **maximal** growth rate is attained, whereby bacteria enter the exponential phase of growth. The acceleration phase lasts for a very short period and for most authors, it is indistinguishable from the exponential phase and some authors prefer to merge it with either the lag or exponential phase.

The Exponential Phase:

The phase during which the growth rate is maximal and constant is termed the exponential or logarithmic growth phase. During this phase, the number of new cells increases exponentially and the cells are in a metabolic condition known as **balanced growth**. In balanced growth, all measurable components of the cell such as protein, DNA, RNA or biomass are manufactured at constant rates relative to each other. During exponential growth, all the cells in the culture are physiologically identical for all practical purposes and the chemical composition of the cells is virtually identical. Cells in exponential growth are typically in their healthiest state and hence are most

desirable for studies of their enzymes or other cell components. The rate of exponential growth for a given species is influenced by environmental conditions (temperature, pH, ionic strength, composition of the culture medium), physiological capabilities as well as by genetic characteristics of the organism itself.

Clinically the following are true of pathogens in exponential phase: (a) There is rapid growth of small numbers to replace the losses to host defenses. (b) Slow growth in log phase may occur in chronic disease states. (c) Rapid growth of pathogens in tissues produces the effects of disease before host defenses mount. (d) Little is known about growth rates during infection. (e) Increases or decreases in bacterial populations can be measured, but these will represent the net results of multiplication and destruction by host defenses.

Exponential growth rapidly depletes the available nutrients, and toxic waste products quickly accumulate. As nutrient concentration decreases, or the culture environment becomes more toxic, growth of cells becomes **unbalanced** and various cellular components are synthesized at different rates relative to one another. These factors cause a decrease in, and ultimately, cessation of cellular division. The exponential growth phase is usually fairly brief, lasting only 4 – 10 hours for most rapidly growing bacteria. Bacteria in this phase of growth are usually sensitive to drugs and chemicals and environmental changes such as those caused by heat, drying and radiation.

The Retardation Phase

This phase follows the exponential phase and represents the point where growth of cells becomes unbalanced due to either depletion of available nutrients or accumulation of toxic wastes or both. Growth rate **decreases** slowly but not at a constant rate. This phase precedes the stationary phase, it actually represents the transition between exponential and stationary phase of growth.

The Stationary Phase

Bacterial cultures enter the stationary phase when the environment can no longer support an increase in cell mass as a result of either one or both of two growth limiting situations: (1) an essential nutrient of the culture medium is used up, or (2) a waste product of the organism accumulates in the medium and inhibits growth. Either way, exponential growth ceases and the population reaches the stationary phase. In the stationary phase, the number of viable cells in the culture remains constant because cells are dying at the same rate that new cells are being produced or the population may simply cease to divide but remain metabolically active. Bacteria in a batch culture may enter stationary phase in response to starvation. Some bacteria respond with obvious

morphological changes such as endospore formation, but many only decrease somewhat in overall size. Because of unbalanced growth preceding the stationary phase, the cells in this phase are not uniform in composition; they are usually smaller than cells in the exponential growth phase and are more resistant to antibiotics, chemicals, heat, radiation, and host defenses. Note that during stationary phase, the **total cell count** slowly increases while the viable count stays constant.

The Death (Decline) Phase

The stationary phase is followed by a period in which the cells gradually die off – the **decline or death phase**. The cells die because they are unable to obtain the energy and nutrients needed to maintain essential function. The death rate increases until it reaches a steady level. Like growth, the rate at which cells die is a function of both the type of cells and the environment. It should be apparent that measuring the death of microorganism is somewhat subjective and may be a function of not providing a proper environment for growth, rather than an actual loss of viability. Thus, we define death as the inability of the organism to multiply when placed into a situation where growth is normally supported and maintained.

Although most bacteria go through this normal growth cycle (A-F) some form spores or cysts, in response to adverse environmental conditions. These bacteria enter dormant state after the stationary phase.

Methods for maintaining Bacteria culture in Exponential Growth Phase – Continuous Culture

Our discussion so far about bacterial population growth centered on closed systems called batch cultures in which nutrients are not renewed nor wastes removed. Bacterial cultures can be maintained in the exponential growth phase for extended periods of time for industrial and research purposes by transferring them repeatedly into fresh medium of identical composition while they are still growing exponentially. This is called continuous culture system. Continuous culture systems grow microorganisms with constant environmental conditions maintained through continual provision of nutrients and removal of wastes. Two devices have been invented for carrying out this process automatically; the chemostat and the turbidostat.

The Chemostat

In the chemostat, both growth rate and cell density of the culture can be controlled independently and simultaneously. Two factors govern growth rate and cell density (cell yield) respectively. These are: (1) the dilution rate, which is the rate at which fresh medium is pumped in and spent medium containing

microorganisms is removed; and (2) the concentration of a limiting nutrient, such as a carbon, vitamin or nitrogen source, present in the sterile medium entering the chemostat vessel.

The device consists of a culture vessel with a mechanism for dripping in fresh medium from a reservoir of sterile medium at a regulated rate. The medium in the culture vessel is stirred by a stream of sterile air. Each drop of fresh medium that enters causes a drop of culture to siphon out. The medium is prepared so that one nutrient limits growth yield. Because one nutrient is limiting, the growth rate is determined by the rate at which new medium is fed into the growth chamber, and the final cell density depends on the concentration of the limiting nutrient. The vessel is inoculated, and the cells grow until the limiting nutrient is exhausted, fresh medium from the reservoir is then allowed to flow in at such a rate that the cells use up the limiting nutrient as fast as it is supplied. Under these conditions, the cell concentration remains constant and the growth rate is directly proportionate to the flow rate of the medium. However, if the flow rate is too fast, the cell concentration decreases because the cells are diluted out of the chemostat by the incoming sterile medium faster than they can reproduce.

The disadvantage of the chemostat is that growing cells are always in a state of semi-starvation for one nutrient and must be grown at less than maximum rate to achieve good regulation. These disadvantages are not present in the turbidostat.

The Turbidostat

This device resembles the chemostat except that the flow of medium is controlled by a photocell mechanism that measures the turbidity (usually optical density) of the culture. When the optical density exceeds a predetermined setting, fresh medium is allowed to flow in. Addition of fresh medium dilutes the culture, causing the optical density to decrease as the cells are washed out in the overflow. Addition of new medium stops when then optical density reaches a preset lower limit. The culture then utilizes the medium to increase its optical density until the process is repeated. The cells in a turbidostat can grow at maximum rate at a constant cell concentration. The growth rate can be controlled in the turbidostat only by varying the nature of the medium or the culture conditions (e.g., temperature).

The turbidostat differs from the chemostat in several ways. The dilution rate in a turbidostat varies rather than remaining constant, and a turbidostat’s culture medium contains all nutrients in excess. That is, none of the nutrients is limiting. The turbidostat operates best at high dilution rates; the chemostat is most stable and effective at lower dilution rates.

Maintenance and Storage of Stock Cultures

Pure cultures of microorganisms can be maintained in **stock cultures** (cultures for storage) for periods varying from weeks to years depending on the organism and the stock-culturing technique employed. Preparing stock cultures is very important since many bacterial strains are difficult to obtain in pure culture, and painstaking experimentation is often required to isolate and characterize many mutants.

Stock cultures of bacteria, fungi and algae are maintained on agar **slants** (aerobes) or in agar **stabs** (anaerobes). An agar slant (also called a slope) provides a large oxygen-exposed surface area for growth whereas the agar in the bottom of a stab creates an oxygen-depleted environment for anaerobes. Some microbes remain viable in refrigerated stock cultures for weeks or months, while other organisms must be transferred periodically to new media. Layering sterile mineral oil over the growth on a slant prolongs the storage life of some organisms.

Stock cultures prepared for long-term storage and for culture collections are either **freeze-dried (lyophilization)** or **stored in liquid nitrogen at -196º C or at -75º C** in a low temperature freezer. Once freeze-dried, cultures of bacteria, fungi, and algae will remain viable for years even when they are stored at room temperature. Similarly, bacterial culture stored in liquid nitrogen at the appropriate temperature remains viable for years.

BACTERIAL CULTURE MEDIA

Research in microbiology depends largely on the ability to grow and maintain microorganisms in the laboratory, and this is possible only if suitable culture media are available. A culture medium is a solid or liquid preparation used to grow, transport, and store microorganisms. Culture media encourage the growth, support, and survival of microorganisms. Essentially, it is a substance designed to create nutritional conditions similar to the natural environment in which the microorganism commonly survives and reproduces. To be effective, the medium must contain all the nutrients the microorganism requires for growth. Specialized media are essential in the isolation and identification of microorganisms, the testing of antibiotic sensitivities, water and food analysis, industrial microbiology, and other activities. Without high-quality media then the possibility of achieving accurate, reproducible, and repeatable microbiological test results is reduced. Although all microorganisms need sources of energy, carbon, nitrogen, phosphorus, sulfur, and various minerals, the precise composition of a satisfactory medium depends on the species one is trying to cultivate because nutritional requirements vary so greatly. Knowledge of a microorganism’s normal habitat often is useful in selecting an appropriate

culture medium because its nutrient requirements reflect its natural surroundings. The media (sing., medium) that are used in microbiology laboratories to culture bacteria are referred to as **artificial media** or synthetic media, because they do not occur naturally; rather, they are prepared in the laboratory.

Culture media can be classified based on several parameters: (1) the chemical constituents from which they are made, (2) their physical nature, and (3) their function.

1. Chemical types of culture media

Culture media can be classified based on chemical constituents into (a) **defined media**, and (b) **complex media**.

(a) Defined media

These are media in which all chemical components are known and well defined. Defined media are prepared by adding precise amounts of highly purified inorganic or organic chemicals to distilled water. Therefore, the exact composition of a defined medium (in both a qualitative and quantitative sense) is known. Defined media are used widely in research, as it is often desirable to know what the microorganism is metabolizing. It can be constructed in the laboratory.

(b) Complex media

Complex media on the other hand contain some ingredients of unknown chemical composition. Complex media contain undefined components such as peptones, meat extract, yeast extract, and digested extracts from animal organs (*e.g.*, hearts, livers, brains). Peptones are protein hydrolysates prepared by partial proteolytic digestion of meat, casein, soya meal, gelatin, and other protein sources. They serve as sources of carbon, energy, and nitrogen. Beef extract and yeast extract are aqueous extracts of lean beef and brewer's yeast, respectively. Beef extract contains amino acids, peptides, nucleotides, organic acids, vitamins, and minerals. Yeast extract is an excellent source of B vitamins as well as nitrogen and carbon compounds.

Such media are very useful, as a single complex medium may be sufficiently rich to meet all the nutritional requirements of many different microorganisms. In addition, complex media often are needed because the nutritional requirements of a particular microorganism are unknown, and thus a complex medium cannot be constructed. They are also used to culture fastidious microbes, microbes with complex nutritional or cultural requirements. Some fastidious microbes may even require a medium containing blood or serum. However, the disadvantage of a complex medium is its imprecise nutritional

composition. That is, although one may know approximately what is in the medium, its exact composition is unknown.

2. Physical types of culture media

Based on physical nature, culture media can be in a liquid form, semi-solid or solidified by an agent such as agar. Liquid media (also known as broths) are contained in tubes and are thus often referred to as tubed media. Solid or semi-solid media are prepared by adding agar to liquid media and then pouring the media into tubes or Petri dishes, where the media solidifies. These 'solid' media are liquid when hot (so that they may easily be poured into the plates) but the medium sets to a gel upon cooling to room temperature. Although both liquid and solidified media are routinely used, solidified media are particularly important because they can be used to isolate different microbes from each other to establish pure cultures. Agar is a complex polysaccharide that is obtained from a red marine alga; it is used as a solidifying agent, much like gelatin is used as a solidifying agent in the kitchen.

Agar is well suited as a solidifying agent for several reasons. One is that it melts at about 90°C but once melted does not harden until it reaches about 45°C. Thus, after being melted in boiling water, it can be cooled to a temperature that is tolerated by human hands as well as microbes. Furthermore, microbes growing on agar medium can be incubated at a wide range of temperatures. Finally, agar is an excellent hardening agent because most microorganisms cannot degrade it.

3. Functional Types of Media

Culture media can also be classified based on their function into

(a) Basic or Routine
(b) Enriched and enrichment
(c) Selective
(d) Differential
(e) Transport
(f) Identification

(a) Basic or Routine Media

These are simple media that will support the growth of microorganisms that do not have special nutritional requirements. Another name for them is general purpose or supportive media because they sustain the growth of many microorganisms. The two common general purpose medium types are nutrient agar or broth and tryptone soya agar or broth.

Uses of Basic/ Routine Media

(a) They are often used in the preparation of enriched media.
(b) They are used to maintain stock cultures of control strains of bacteria.
(c) Finally, they are used for sub-culturing pathogens isolated from primary culture of differential or selective media prior to performing biochemical and serological identification tests.

(b) Enriched Media

These are media that are enriched with whole blood, lyzed blood, serum, peptone extracts, special extracts, or vitamins to support / encourage the growth of pathogens with exacting growth requirements (i.e., organisms that require additional nutrients or growth stimulants (fastidious organisms) e.g., *H. influenzae*, *Neisseria* species and some *Streptococcus* species). They are solid media and often used for specimen collected from sites which are normally sterile to ensure the rapid multiplication of a pathogen which may be present only in small numbers. It is usually prepared by adding extra nutrients to a medium called nutrient agar. Examples of enriched media are blood agar, serum agar, yeast extract agar, vitamin agar and tryptone soya media used to produce a better and more rapid growth of a wide range of pathogens. Blood agar is both an enriched and differential medium.

The term **enrichment** is used to describe a fluid medium that increases the numbers of a microorganism by containing enrichments, and, or substances that inhibit the multiplication of unwanted bacteria. Examples include Rappaport-Vassiliadis broth used for Salmonella species, Selenite F broth used as an enrichment medium for Salmonellae in faeces or urine prior to sub-culturing on Xylose Lysine Deoxycholate agar or other enteric selective medium. Another example is Alkaline Peptone Water which acts as an enrichment and transport medium for *Vibrio cholerae*. Both enriched and enrichment media contain a rich supply of special nutrients that promotes the growth of fastidious organisms.

(c) Selective Media

These are solid media which contain substances (e.g., bile salt, dyes, antibiotics, and other chemicals) that inhibit the growth of microorganisms to allow the growth of another to be more clearly demonstrated. Examples of such dyes include basic fuchsin and crystal violet. A selective medium is used when culturing a specimen from a site having a normal microbial flora to prevent unwanted contaminant overgrowing a pathogen. Examples include, Eosin methylene blue (EMB) agar used for isolating Gram-negative bacteria e.g., E. coli. It inhibits the Gram positives bacteria; Xylose Lysine Deoxycholate agar is used for isolating Salmonellae and particularly Shigellae from faecal specimens. It contains bile salts that inhibit the growth of many faecal commensals.

In recent years, antimicrobials have been increasingly used as selective agents in culture. Examples of antimicrobial selective media include Cetrimide Agar used to isolate *Pseudomonas* species e.g., *Pseudomonas aeruginosa* from a mixed bacterial flora and the Modified New York city (MNYC) medium for isolating *Neisseria gonorrhoeae* from urinogenital specimens.

Other ways of selecting organisms

(1) Incubation condition or atmosphere may be used to select organism e.g. *P. aeruginosa* is inhibited by anaerobic conditions.
(2) Also, the pH of a medium may make it selective for a particular organism e.g. *V. cholerae* can be isolated on an alkaline medium such as Thiosulphate citrate bile salt sucrose agar (TCBS).
(3) Temperature may also help to select an organism e.g. *Listeria monocytogenes* can grow at 4^{o}C whereas other organisms are inhibited. Growth however is slow.

(d) Differential (indicator) Media

These are media to which indicators, dyes or other substances are added to differentiate microorganisms and even permit tentative identification of microorganisms based on their biological characteristics e.g., Thiosulphate Citrate Bile salt Sucrose agar contains the indicator bromothymol blue which differentiates sucrose fermenting from non-sucrose fermenting Vibrio species, while MacConkey agar contains neutral red indicator which differentiates lactose fermenting from non-lactose fermenting Gram-bacteria. Mannitol salt agar is used to screen for *Staphylococcus aureus;* not only will *S. aureus* grow on MSA, but it turns the originally pink medium to yellow due to its ability to ferment mannitol.

Most, but not all differential media distinguish between bacteria by an indicator which changes colour when acid is produced following carbohydrate fermentation. Blood agar, however, can also be described as a differential medium when it differentiates haemolytic from non-haemolytic bacteria.

Many culture media are both differential and selective e.g., Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar. MacConkey Agar, Xylose Lysine Deoxycholate (XLD) agar and DCA. Enriched media may also be made selective and/or differential e.g., Crystal violet blood agar is an enriched, selective and differential medium for *Streptococcus pyogenes*.

(e) Transport Media

These are mostly semi-solid media that contain ingredients to prevent the overgrowth of commensals and ensure the survival of aerobic and anaerobic microbes when specimens cannot be cultured soon after collection. Their use is particularly important when transporting microbiological specimens from health centres to the district microbiology laboratory. Examples of transport media include **Cary-Blair Medium** for preserving enteric pathogens and **Amies Transport Medium** for ensuring the viability of gonococci and other pathogens in specimens collected on swabs.

(f) Identification Media

These are media to which substrates or chemicals are added to help identify bacteria isolated on primary culture e.g., Peptone water sugars, Urea broth, Urea agar and Kligler iron agar. Organisms are mainly identified by a change in the colour of the medium and or the production of gas. Organisms used to inoculate identification media must be first isolated in a pure culture.

Preparation and Storage of Bacteriological Media

Most essential culture media are available commercially in ready-made **dehydrated** form. Dehydrated media is hygroscopic and thus becomes unfit for use when exposed to moisture. Whenever possible, it is advisable to use the ready-made standardized dehydrated media in order to ensure good performance and reproducibility.

However, where such ready-made media are not available, researchers have to prepare them themselves, using standard formula outlined in the media manual. Even when the media is available in a ready-made form, preparing it to the desired form wherein the necessary bacteriological procedures will be carried out requires carefulness, training and experience.

In order to prepare a culture media that would be successfully used for routine bacteriological procedures, each of the following general steps must be performed correctly:

1. Weighing and dissolving
2. Sterilization
3. Addition of heat-sensitive ingredients
4. Dispensing
5. Storage.

1. Weighing and dissolving

The following apply when weighing and dissolving culture media:

(a) Use only ingredients that are suitable for microbiological use (Bacteriological Quality), i.e. it contains no substance harmful to microorganisms.
(b) Weigh accurately, using a balance with a high sensitivity; always weigh in a cool, clean and dry atmosphere.
(c) When weighing dehydrated media, weigh rapidly and make sure the tops of stock bottles are replaced immediately and tightly.
(d) Once the ingredients are weighed, do not delay in making up the medium.
(e) Use completely clean glassware, plastic or stainless-steel equipment that has been rinsed in clean water. The container in which the medium is prepared should have a capacity of at least twice the volume of the medium being prepared.
(f) Use distilled water from a glass still or deionized water provided the exchange resins do not contain substances inhibitory to bacteria.
(g) Add the powdered ingredients to the water and stir to dissolve. Do not shake a medium but mix by continuous stirring or by rotating the flask.
(h) When heating is required to dissolve the medium, stir while heating and control the heat to prevent boiling and foaming which can be dangerous and can damage the medium, e.g., DCA or TCBS agar. Also, overheating a medium can alter its nutritional and gelling properties, and also its pH.

(i) Autoclave a medium only when the ingredients are completely dissolved. Always autoclave at the correct temperature and for the time specified.

2. Sterilizing culture media

Always sterilize a medium at the correct temperature for the correct length of time. If using a dehydrated medium, always follow exactly the manufacturer's instruction. The methods commonly used to sterilize culture media are: (a) autoclaving, (b) steaming at 100º C and (c) filtration.

(a) Autoclaving is used to sterilize most agar and fluid culture media. It ensures the destruction of bacterial endospores as well as vegetative cells by coagulating and denaturing microbial proteins and enzymes.
(b) Steaming at 100º C is used to sterilize media containing ingredients that would be broken down or inactivated at temperatures over 100º C. Examples include Selenite F broth and Cary-Blair transport medium. Steaming is also used to re-melt previously bottled sterile agar media.

(c) Filtration is mainly used to sterilize additives that are heat-sensitive and cannot be autoclaved, or less stable substances that need to be added to a sterile medium immediately before it is used. Examples include serum and solutions containing urea and certain carbohydrates.

(c) Addition of heat-sensitive ingredients

If the ingredient has been refrigerated, e.g., blood or serum, it must be allowed to warm to room temperature before being added to a molten agar medium. The heat-sensitive ingredient should be added aseptically when the medium has cooled to about 50º C. A medium to which a heat-sensitive substance has been added must not be autoclaved. It should be distributed into plates immediately unless further heating is required (e.g., chocolate agar).

(d) Dispensing culture media

Media should be dispensed in a clean draught-free room. When dispensing sterile media, sterile containers and an aseptic technique must be used. The following general procedure is used in dispensing agar media in Petri dishes:

(a) Lay out the sterile Petri dishes on a level surface.

(b) Mix the medium gently by rotating the flask or bottle. Flame-sterilize the neck of the flask or bottle and pour about 20 ml of medium in each Petri dish. If air bubbles enter while pouring, rapidly flame the surface of the medium before gelling occurs.

(c) When the medium has gelled, and cooled, stack the plates and seal them in plastic bags to prevent loss of moisture. For fluid media, most are dispensed in screw-cap bottles or tubes, and then sterilized. Sterile agar media are similarly dispensed in tubes and bottles. In this case, they are dispensed aseptically in sterile screw-cap bottles or tubes, and allowed to set in a sloped or upright position (agar deeps).

(e) Storage of culture media

Dehydrated culture media and dry ingredients such as agars, peptones, bile salts and carbohydrates (solid form) should be stored at an even temperature in a cool dry place away from direct light. Container tops must be tight-fitting.

Additives such as blood, serum, urea, antimicrobials in solid form and carbohydrate solutions require storage at 2 - 8º C. All additives should be allowed to warm to room temperature before being used.

Plates or culture media should be stored at 2-8^{o} C preferably in sealed plastic bags to prevent loss of moisture. Most media in a screw-cap tubes or bottles can be stored at room temperature. Prepared media must be protected from direct light, especially sunlight which can cause some media to become bactericidal.

DEVELOPMENT (EVOLUTION) OF PURE CULTURE TECHNIQUE

The origins of microbiological culture media can be traced to the 19th Century when the science of bacteriology was just beginning. During this pioneering time bacteriologists attempted, with variable success, to grow microorganisms either directly using the food or material on which the microorganism had first been observed or some compound thereof. These were primarily beef-based broths of unknown and variable composition. The first person to cultivate microorganisms on a growth medium, with a degree of reproducibility, was Louis Pasteur (1822-1885). Pasteur fashioned a media of yeast, ash, candy sugar and ammonium salts in 1860. This medium contained the basic requirements for microbial growth: nitrogen (ammonium salts), a carbon source (sugar) and vitamins (ash). In developing the media Pasteur made some important observations: that particular chemical features of the medium can promote or impede the development of any one microorganism and that competition occurs among different microorganisms for the nutrients contained in the media, which can lead to some species outgrowing and dominating a culture.

Robert Koch (1843-1910) discovered that broths based on fresh beef serum or meat extracts (called ‘broth’ for liquid culture medium being analogous to broth or soup) produced optimal growth. Koch’s groundbreaking work led to his recognition as ‘The Father of Culture Media’.

In 1881 Koch initial attempts to isolate cultures pathogens egg albumen, starch paste and an aseptically cut slice of potato proved relatively poor at recovering pathogenic bacteria. The potato slices were overgrown with moulds and he discovered that broth media cannot be used for isolation of pure cultures. His search for solid media alternatives led to the isolation of *Bacillus anthracis,* (the causative agent of anthrax) for the first time, in 1882, which represented a major step-forward in disease control. In his quest for solid media, Koch developed a meat extract with added gelatin (a colourless substance derived from the collagen inside animals' skin and bones). The resulting ‘nutrient gelatin’ was poured onto flat glass plates which were inoculated and placed under a bell jar.

Although nutrient gelatin was a major advance, gelatin had two major disadvantages as a gelling agent: It turned from a gel to a liquid at 25^{o} C upwards – preventing plates from being incubated at higher temperatures (many

common bacteria, mesophilic organisms like those of the genera Staphylococcus, have an optimal growth range of 30-35° C). The temperature sensitivity also caused the medium to melt in the summer months. It was hydrolysed by gelitinase – an enzyme produced by most proteolytic microorganisms into various sub-compounds (polypeptides, peptides, and amino acids) rendering the medium useless. A year later, Koch's attempts at a nutrient medium were advanced.

In 1882 Fannie Hesse (1850-1934), the wife of Dr. Walther Hesse (1846-1911) (who was Koch's research assistant), suggested replacing gelatin with agar. Fannie Hesse had been inspired by the use of agar to prepare fruit jams and jellies.

Agar (or 'agar-agar') is a water soluble polysaccharide derived from red-purple seaweeds (belonging to the genus *Gelidium* and *Gracilaria*). Agar proved to be a superior gelling agent. Agar has physical properties which could be readily adapted for bacteriology. Agar melts when heated to around 85° C, and yet when cooled it does not form a gel until it reaches 34-42° C (a physical property called hysteresis). Agar is also clearer than gelatin and it resists digestion by bacterial enzymes. The use of agar allows the creation of a medium that can be inoculated at 40° C in its cooled molten state and yet incubated up 60° C without melting (a useful characteristic when examining for thermophiles). Typically, a 1-12% final concentration of agar is used for solidifying culture media.

The ability to grow bacteria on solid media was to prove a major milestone in the development of bacteriology. The formation of bacteria on solid media led Koch to use the word '**colony**' to describe the pure and discrete growth.

A further important development for the manufacture of solid media in the development of pure culture occurred in 1887 when Julius Richard Petri (1852-1921), a worker in Koch's laboratory, produced a new type of culture dish for media. This was the Petri dish. The key design feature of Petri dish was the use of an overhanging lid, which was in place to keep contaminants out from medium inside. Petri dish is still very important in microbiology till date.

For many years glass dishes were used, mainly until the mid 1960s, where advances with injection molding technology lead to Petri dishes being manufactured out of clear polystyrene plastic. These dishes were very similar to the first, glass Petri dishes.

Agar provides the structure for solid microbiological media but it does not provide the nutrients necessary for bacteria to grow. In 1884 Fredrick Loeffler added peptone and salt to Koch's basic meat extract formulation which was insufficient in amino-nitrogen to allow optimal growth of a range micro-organisms. The peptone he used was an enzymatic digest of meat which in the

19th Century was a pharmaceutical product prescribed for nutritional disorders. This peptone added amino-nitrogen, while the salt raised the osmolarity of the medium.

From laboratory to mass production

By the 1890s culture media had developed to form familiar to microbiologist of the 21st Century: clearer broths; solid media in Petri dishes; and the widespread use of peptones and agar. For example, Loeffler made a significant advance by developing nutrient broth to cultivate *Corynebacterium diphtheria*. The formulation of the nutrient broth is still widely used today (Loeffler's Medium). It became increasingly apparent that there was a gap in market place for mass produced culture media. The development of commercially produced culture media originated with the meat industry, whereby hitherto discarded by-products from the manufacture of meat products were used to produce culture media. The most prominent example of this was the German Baron Justus von Liebig who, in the 1865 established a company - the Liebig Extract of Meat Company (LEMCO) to manufacture and sell Liebig's extract of meat.

Liebig developed his meat extract as a food source. His initial motivation was to provide food source, stable at room temperature, for the growing malnourished poor people in central Europe. This became company’s most famous product: the Oxo cube. It was only later that a use for the waste product from the manufacture of Oxo cubes was found: to manufacture microbiological culture media. By 1924, the products of OXO Medical Division of LEMCO were sold to hospitals and laboratories.

The driving force for the large scale production of microbiological media and so many scientific innovations in the UK was World War II. Oxo Limited became the main provider of microbiological culture media to UK the Public Health Laboratory Service. The ability of Oxo to provide media on a large scale was accelerated by the development of dehydrated culture media, whereby media was preserved for long periods by removing water. Low amounts of water resulted in the media powder having a low water activity which reduced the possibility of spoilage occurring. In the USA, similar movements towards mass production occurred during the war undertaken by the American Agar Company of San Diego, California and by the Digestive Ferments Company (Difco).

The Twentieth Century: further advances

At the start of the 20th Century, further advances in culture media and pure culture technique occurred. Most of the media used during the 19th Century was non-selective and was designed to grow a range of bacteria. The first step towards diagnostic media production took place in 1888 when

Martinus Beijerinck (1851 - 1931) developed an elective medium (one which uses nutritional requirements to limit what can grow on a plate). Beijerinck wanted to isolate the root nodule bacterium Rhizobium, which is capable of fixing atmospheric nitrogen. To do this he designed a medium containing no nitrogenous compounds. This inhibited the growth of non-nitrogen fixing microorganisms and produced a pure culture of Rhizobium. Beijerinck went on to use another elective medium, based on the ability of certain microorganisms to use CO_2 as a carbon source under anaerobic conditions, to isolate the first pure culture of sulphur-oxidizing bacterium *Thiobacillus denitrificans* in 1904.

Although chemicals, such as dyes, had been known to have antimicrobial effects since 1885, (when Paul Ehrlich published work on the inhibitory effect of arsenic compounds on syphilis) they were not incorporated into media formulations until the first selective media were developed in the 1900s. Early selective agents tended to be chemicals and dyes used for other purposes within the laboratory that were found, by chance, to have the ability to inhibit certain microorganisms. Some of the most important developments included:

(a) 1905: Alfred MacConkey, used bile salts to select for lactose fermenting bacteria in faecal samples. The level of conjugation in the bile salts determines its selectivity profile: conjugated bile salts are less inhibitory and allow the growth of Staphylococci and Enterococci; while more disassociated salts such as desoxycholate are much more selective, only allowing growth of Enterobacteriacae.

(b) 1912: Churchman showed that derivatives of triphenylmethane such as gentian violet and brilliant green dyes were inhibitory to bacteria, particularly Gram positives; and crystal violet causes some inhibition of fungi.

(c) 1923: Muller described a medium using iodine and sodium thiosulphate which react together to form tetrathionate. The selectivity of tetrathionate depends on whether or not an organism possesses the enzyme tetrathionase. Salmonellae and Proteus species possess the enzyme, so can grow in the presence of tetrathionate.

Another important development was the addition of antibiotics to media. It was in the 1960s that antibiotics were used in culture media, for the first time, as selective agents. For example, Thayer-Martin produced in 1964, a formulation for the isolation of *Neisseria gonorrhoeae* and *N. meningitides*, using a mixture of vancomycin, colistin and trimethoprim. This was one of the most widely documented early examples of antibiotics being used in a selective medium. In parallel with the development of selective media, diagnostic media was produced in the early 20th Century. The main driver for this was the

diagnostic importance of haemolytic reactions. In 1919 James Brown used a blood agar to study the haemolysis reactions of the genus *Streptococcus* and from this he was able to differentiate alpha, beta and gamma haemolysis, an important step for differentiating and identifying different species of *Staphylococcus* in medical diagnosis.

Continuing developments

The discovery of new microorganisms presents new challenges and this requires new isolation methods. For example, in 1977 Joseph McDade and Charles C. Shepard identified *Legionella pneumophilia* as the pathogen which caused Legionnaires disease which required the development of new media both for clinical and water testing applications. Other notable developments include a special medium for recovering bacteria from water systems (Reasoner and Geldrich's R2A agar). In addition, in 1983 Barry Marshall demonstrated that isolates from gastric and duodenal ulcers all contained a *Campylobacter*-like organism later called *Helicobacter pylori*. More recently, in 2002 the first Vancomycin Resistant *Staphylococcus aureus* (VRSA) were found in Michigan and Pennsylvania, in the USA, which led to the development of differential media for VRSA. With another more recently identified bacterium a special chromogenic media was developed to specifically detect the emerging pathogen *Enterobacter sakazakii* from infant formula milk.

Specialist media like chromogenic media represents another relatively recent development. Chromogens are molecules designed to mimic metabolic substrates which are colourless until they are cleaved by the target enzyme. Once cleaved the molecule becomes both insoluble and coloured, and so builds up within the cell. This means that colonies of an organism which possess the enzyme can be easily differentiated from those that do not. By designing a selective base medium and adding chromogenic substrates, media can be designed that allow differentiation and identification of groups of organisms. A large number of chromogenic media are now available for organisms as wide ranging as *E. coli* and coliforms, *Salmonella*, *Listeria monocytogenes*, urinary tract pathogens, *Clostridium perfringens*, and *Candida* species.

Media manufacture in the Twenty-First Century displays continuities and similarities with the pioneering manufacturers of the past. The main ‘active’ ingredient of culture media remains peptones. Today peptones are still largely manufactured from meat (such as liver, heart and veal); but also from milk and vegetable sources (of which pea sources are the most common). Peptones are hydrolysed using either acid or enzymes.

For solid media, agar continues to be produced from seaweed. Developments in processing techniques allow agars with different characteristics to be produced. For example, agars with a low or high

temperature gelling or low syneresis (that is, moisture loss). A second important criteria is that solid media has a constant gel strength which has dramatic effect on colony morphology and growth. High gel strength media will grow small colonies because the flow of nutrients and removal of toxins is reduced. Low gel strength media will allow the growth of larger colonies, but can be difficult to' streak out' the colony.

In the bacteriology laboratory of today there are three main types of culture media: (i) Natural or empirical culture media, (ii) Synthetic or defined culture media, and (iii) Living media.

Initially all types of media, starting with Koch and his researchers, produced belonged to what is now described as 'natural' or 'empirical' culture media. Such media are commonly used today. Most empirical media contain only peptone as the major ingredient (a trypsinized or hydrolysed protein). Today such empirical media, including simple solutions of peptones or yeast extracts, are sometimes loosely included in the general terms nutrient broth or nutrient solution.

With synthetic or defined culture media, such media consist wholly of dilute, reproducible solutions of chemically pure, known inorganic and/or organic compounds. The formulation and use of these media requires an exact knowledge of the nutritional requirements of the microorganism to be cultivated.

A third type of culture media is 'living' culture media. Living Culture Media are made up of groups of living cells as tissues, or callus or an organ used for growing viruses, rickettsias and so forth. Chick embryos are commonly used for cultivation of viruses. Yeasts, moulds and bacteria, if found as contaminants, are able to multiply in the fluids of the chick embryos as in a culture tube.

Conclusion

Despite the advent of rapid microbiological methods, culture media remains the fundamental tool for the bacteriologist, whether for undertaking research or for regular quality control tasks like environmental monitoring. Many practitioners of microbiology remain uninformed about the origins of their primary 'tool', which to some extent is unsurprising given that few detailed accounts exist.

METHODS OF MEASURING GROWTH

Bacterial population growth can be measured on the basis of total number of cells, number of viable cells or cell mass of the culture. Alternatively, one can measure the increase in a specific cellular constituent such as DNA or

protein, but such measurements are difficult and are made only in special circumstances.

1. **Total Cell Count:**

This involves counting both viable and dead cells. Two methods are generally adopted:

(a) Direct microscopic counts,

(b) Electronic counts

(a) Direct microscopic counts

Direct microscopic counting is a quick and relatively easy method of determining approximate numbers of microorganisms in a liquid culture. Special counting chambers such as the Petroff-Hausser chamber are used. The chamber has measured grids marked on the surface and a cover slip that is held at a precise distance over the chamber. The space between the slide and the cover slip is filled with a fluid containing microbial cells and the number of cells in a specified number of squares of the grid is counted. Because the volume of fluid over each square is known, the number of microorganisms per cubit volume can be determined.

(b) Electronic counts

Total cell counts of bacterial and mammalian cultures can also be made with an electronic particle counter. The one in common use is the Coulter counter, invented by J.A. Coulter. This counter uses changes in electrical impedance created as the bacteria are drawn through a small opening in an electric field to count the bacteria. Although relatively accurate, the instrument is expensive and requires that the bacterial suspending fluid absolutely clean and free of all dust or particles which, like bacteria, would be counted as they pass through the electric field.

2. Viable Cell Count

The number of viable cells in a culture can be ascertained by determining the number of colony-forming units (CFU) by plate counting techniques which include spread-plate method, Miles and Misra method, and Pour-plate method.

(a) Spread-plate method

One method of obtaining plate counts is the spread-plate method. For this procedure, the fluid to be examined is spread evenly over the surface of a bacterial growth medium. After incubation for 24 to 48 hrs, colonies develop where each viable bacterium was deposited. The number of colonies is then multiplied by the reciprocal of a dilution factor to give the original concentration of cells per milliliter.

(b) Miles and Misra method

Another method of obtaining plate count is Miles and Misra method**.** It is a slight modification of spread-plate method. Here the fluid to be examined or counted is dropped on the surface of the an overdried agar medium in small volumes of 0.015 ml, which is allowed to spread over a small area before percolating into the medium. After percolation, the cells are left on the surface of the medium to grow into colonies during 24 hrs. incubation at 37^{o} C. Each drop of fluid on the agar medium is a replicate reading. The issuing colonies are counted and divided by the number of drops made on the agar surface to obtain average colony count per drop (0.015 ml). Thereafter, calculate the CFU/ml by multiplying the average colony count per drop with the reciprocal of a dilution factor divided by the volume plated (0.015 ml).

(c) Pour-plate method

Another process is the pour-plate method. In this case, a measured volume, usually 0.1 or 1.0ml of test fluid is mixed with melted agar medium that has been cooled to about 48° C. The agar is then poured into a sterile Petri dish where it solidifies. The bacteria are trapped in the agar and each viable organism develops into a colony after incubation for 1 to 2 days.

The number of bacteria is so great in many samples that if undiluted samples were plated directly the many colonies could fuse to form a solid layer of bacteria and the number could not be determined. It is thus necessary in most cases to dilute the sample before plating and the ten-fold serial dilution technique is usually employed. The colonies are counted on those plates that produce between 30 and 300 colonies because these numbers have been shown to give the most reliable indication as to the correct number of cells. The average number of colonies (CFUs), usually on duplicate plates is determined. The number of viable bacteria per milliliter of the initial culture can be calculated from the average CFUs and the known dilution factors.

The major disadvantages of the colony plating technique are (1) the incubation period is lengthy, (2) sterile dilution media, pipettes and plates are required, and (3) samples and dilution errors occur.

3. Cell Mass

Cell mass of microbial cultures could be rapidly determined by measuring the degree of turbidity of the culture using an instrument called spectrophotometer. This instruments measures how much light passes through a solution or suspension and registers the result on a scale of % transmittance or absorbance (often called optical density). The amount of turbidity is directly

proportional to the mass of cells present and hence directly proportional to the number of cells. Therefore, the higher the turbidity of the culture (corresponding to increased number of cells due to bacterial growth), the lower the % light transmittance and the higher the optical density.

Although optical density measurement is a rapid and accurate method of measuring growth, it presents some disadvantages: (1) large cell contribute more to the optical density than to small cells, (2) at least 10^6 cells per milliliter are required before a reliable reading can be obtained, (3) cells can aggregate and settle out before the measurement is made and (4) both non-living and living cells are counted. Problem 4 can be overcome by standardizing the optical density measurement with viable cell counts.