UNIT – III MICROBIAL GROWTH AND PHYSIOLOGY

SATHYABAMA UNIVERSITY **FACULTY OF BIO AND CHEMICAL ENGINEERING**

SBT1103	MICROBIOLOGY	L	T	P	Credits	Total Marks
		3	0	0	3	100

COURSE OBJECTIVES

- To enable students to learn about the principles of Microbiology to emphasize structure and biochemical aspects of various micro organisms.
- To know the control and preventive measures of microbial infections and environmental pollutions.

UNIT 1 INTRODUCTION TO MICROBIOLOGY **11 Hrs.**

Introduction, History and scope of microbiology, Contributions of Leewenhoek, Pasteur, Koch, Jenner and Fleming, Microbial classification: Classical and Current systems, Methods of identifying microbes.

Basics of Microscopy, Staining: simple, differential (Gram staining, Acid fast staining), special staining (flagella,capsule,endospore)

UNIT 2 MICROBIAL STRUCTURE AND REPRODUCTION **9 Hrs.**

Morphology and Reproduction: Bacteria - General structure and forms, Reproduction methods - Fission, budding and sporulation, Virus - TMV, HIV & T4 bacteriophage - lytic, lysogenic cycle, Fungi - Fungal morphology - Mycelial and yeast forms - sexual and asexual Reproduction, Actinomycete

UNIT 3 MICROBIAL GROWTH AND PHYSIOLOGY **7 Hrs.**

Microbial Growth and Nutrition, Types of media - Based on Consistency, Nutritional components, Funtional uses and application, Microbial types based on nutrition, Growth of microbes in culture - Pure culture techniques, Batch & Continous - Growth curve - Enumeration methods , Types of fungal growth media. Aerobic and Anaerobic metabolism of sugars, mixed acid fermentation.

UNIT 4 CONTROL OF MICROORGANISMS **9 Hrs**

Definitions of frequently used terms - Pattern or Rate of Microbial Death, Physical methods of Microbial Control: Heat (Moist & Dry), Low temperature, Filtration, High pressure, Desiccation, Osmotic pressure, Radiation. Chemical methods of Microbial Control: Liquids - Alcholos, Aldehydes, Phenolics, Halogens - Heavy metals, Surface active agents & Dyes, Gases - Formaldehyde, Ethylene Oxide, Plasma - Physico-chemical methods - Chemotherapeutic agents - Evaluation of effectiveness of antimicrobial agents. Difference between cleaning - sanitizing - sterilizing agents. Moist heat sterilization: D, Z and F Values and significance.

UNIT 5 APPLICATIONS OF MICROBIOLOGY **9 Hrs.**

Microbial ecology: Microbe-Microbe interaction - Mutualism, Commensalism, Altruism, Microbe - host interactions - Colonization and Infection- Causes and Transmission of Infectious Diseases, Emerging and re-emerging infectious diseases - Mechanism and examples, Multidrug resistance - MRSA, Diagnostic Microbiology, Childhood and adult vaccinations - MMR, Polio, Rabies etc, bioterrorism agents, Biofilm - Quorum sensing,

Max. 45 Hours.

TEXT / REFERENCE BOOKS

1. Pelczar, Jr E.C.S Chan and noel R.Krieg, Microbiology, 5th edition Tata McGrawHill -2006
2. Joanne M. Willey, Linda Sherwood, Christopher J. Woolverton, Prescott's Microbiology, 8th Edition, McGraw-Hill Higher Education, 2008
3. Jawetz, Melnick and Adelberg's Medical Microbiology . McGraw-Hill Medical, 2007
4. University of South Carolina School of Medicine (http://pathmicro.med.sc.edu/book/bact-sta.htm)

END SEMESTER EXAMINATION QUESTION PAPER PATTERN

Max Marks : 80 **Exam Duration : 3 Hrs.**

PART A : 10 questions of 2 marks each - No choice **20 Marks**

PART B : 2 questions from each unit of internal choice; each carrying 12 marks **60 Marks**

Bacterial culture media

One of the most important reasons for culturing bacteria in vitro is its utility in diagnosing infectious diseases. Isolating a bacterium from sites in body normally known to be sterile is an indication of its role in the disease process. Culturing bacteria is also the initial step in studying its morphology and its identification. Bacteria have to be cultured in order to obtain antigens from developing serological assays or vaccines. Certain genetic studies and manipulations of the cells also need that bacteria be cultured in vitro. Culturing bacteria also provide a reliable way estimating their numbers (viable count). Culturing on solid media is another convenient way of separating bacteria in mixtures.

History:

Louis Pasteur used simple broths made up of urine or meat extracts. Robert Koch realized the importance of solid media and used potato pieces to grow bacteria. It was on the suggestion of Fannie Eilshemius, wife of Walther Hesse (who was an assistant to Robert Koch) that agar was used to solidify culture media. Before the use of agar, attempts were made to use gelatin as solidifying agent. Gelatin had some inherent problems; it existed as liquid at normal incubating temperatures (35-37^{o}C) and was digested by certain bacteria.

Composition of culture media:

Bacteria infecting humans (commensals or pathogens) are chemoorganoheterotrophs. When culturing bacteria, it is very important to provide similar environmental and nutritional conditions that exist in its natural habitat. Hence, an artificial culture medium must provide all the nutritional components that a bacterium gets in its natural habitat. Most often, a culture medium contains water, a source of carbon & energy, source of nitrogen, trace elements and some growth factors. Besides these, the pH of the medium must be set accordingly. Some of the ingredients of culture media include water, agar, peptone, casein hydrolysate, meat extract, yeast extract and malt extract.

Classification:

Bacterial culture media can be classified in at least three ways; Based on consistency, based on nutritional component and based on its functional use.

1) Classification based on consistency:

Culture media are liquid, semi-solid or solid and biphasic.

A) Liquid media: These are available for use in test-tubes, bottles or flasks. Liquid media are sometimes referred as "broths" (e.g nutrient broth). In liquid medium, bacteria grow uniformly producing general turbidity. Certain aerobic bacteria and those containing fimbriae (Vibrio & Bacillus) are known to grow as a thin film called 'surface pellicle' on the surface of undisturbed broth. *Bacillus anthracis* is known to produce stalactite growth on ghee containing broth. Sometimes the initial turbidity may be followed by clearing due to autolysis, which is seen

in penumococci. Long chains of Streptococci when grown in liquid media tend to entangle and settle to the bottom forming granular deposits. Liquid media tend to be used when a large number of bacteria have to be grown. These are suitable to grow bacteria when the numbers in the inoculum is suspected to be low. Inoculating in the liquid medium also helps to dilute any inhibitors of bacterial growth. This is the practical approach in blood cultures. Culturing in liquid medium can be used to obtain viable count (dilution methods). Properties of bacteria are not visible in liquid media and presence of more than one type of bacteria can not be detected.

B) Solid media: Any liquid medium can be rendered by the addition of certain solidifying agents. Agar agar (simply called agar) is the most commonly used solidifying agent. It is an unbranched polysaccharide obtained from the cell membranes of some species of red algae such as the genera Gelidium. Agar is composed of two long-chain polysaccharides (70% agarose and 30% agarapectin). It melts at 95°C (sol) and solidifies at 42°C (gel), doesn't contribute any nutritive property, it is not hydrolyzed by most bacteria and is usually free from growth promoting or growth retarding substances. However, it may be a source of calcium & organic ions. Most commonly, it is used at concentration of 1-3% to make a solid agar medium. New Zealand agar has more gelling capacity than the Japanese agar. Agar is available as fibres (shreds) or as powders.

C) Semi-solid agar: Reducing the amount of agar to 0.2-0.5% renders a medium semi-solid. Such media are fairly soft and are useful in demonstrating bacterial motility and separating motile from non-motile strains (U-tube and Cragie's tube). Certain transport media such as Stuart's and Amies media are semi-solid in consistency. Hugh & Leifson's oxidation fermentation test medium as well as mannitol motility medium are also semi-solid.

D) Biphasic media: Sometimes, a culture system comprises of both liquid and solid medium in the same bottle. This is known as biphasic medium (Castaneda system for blood culture). The inoculum is added to the liquid medium and when subcultures are to be made, the bottle is simply tilted to allow the liquid to flow over the solid medium. This obviates the need for frequent opening of the culture bottle to subculture.

Besides agar, egg yolk and serum too can be used to solidify culture media. While serum and egg yolk are normally liquid, they can be rendered solid by coagulation using heat. Serum containing medium such as Loeffler's serum slope and egg containing media such as Lowenstein Jensen medium and Dorset egg medium are solidified as well as disinfected by a process of inspissation.

2) Classification based on nutritional component:

Media can be classified as simple, complex and synthetic (or defined). While most of the nutritional components are constant across various media, some bacteria need extra nutrients. Those bacteria that are able to grow with minimal requirements are said to non-fastidious and those that require extra nutrients are said to be fastidious. Simple media such as peptone water, nutrient agar can support most non-fastidious bacteria. Complex media such as blood agar have ingredients whose exact components are difficult to estimate. Synthetic or defined media such as Davis & Mingioli medium are specially prepared media for research purposes where the composition of every component is well known.

3) Classification based on functional use or application:

These include basal media, enriched media, selective/enrichment media, indicator/differential media, transport media and holding media.

A) Basal media are basically simple media that supports most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar considered basal medium.

B) Enriched media: Addition of extra nutrients in the form of blood, serum, egg yolk etc, to basal medium makes them enriched media. Enriched media are used to grow nutritionally

exacting (fastidious) bacteria. Blood agar, chocolate agar, Loeffler's serum slope etc are few of the enriched media.

C) Selective and enrichment media are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria. While selective media are agar based, enrichment media are liquid in consistency. Both these media serve the same purpose. Any agar media can be made selective by addition of certain inhibitory agents that don't affect the pathogen. Various approaches to make a medium selective include addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these.

D) Enrichment media are liquid media that also serves to inhibit commensals in the clinical specimen. Selenite F broth, tetrathionate broth and alkaline peptone water are used to recover pathogens from fecal specimens.

E) Differential media or indicator media: Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony colour. Various approaches include incorporation of dyes, metabolic substrates etc, so that those bacteria that utilize them appear as differently coloured colonies. Such media are called differential media or indicator media. Exmples: MacConkey's agar, CLED agar, TCBS agar, XLD agar etc.

F) Transport media: Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms or commensals. This can be achieved by using transport media. Such media prevent drying (desiccation) of specimen, maintain the pathogen to commensal ratio and inhibit overgrowth of unwanted bacteria. Some of these media (Stuart's & Amie's) are semi-solid in consistency. Addition of charcoal serves to neutralize inhibitory factors. Cary Blair medium and Venkatraman Ramakrishnan medium are used to transport feces from suspected cholera patients. Sach's buffered glycerol saline is used to transport feces from patients suspected to be suffering from bacillary dysentery.

G) Anaerobic media: Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation -reduction potential and extra nutrients. Media for anaerobes may have to be supplemented with nutrients like hemin and vitamin K. Boiling the medium serves to expel any dissolved oxygen. Addition of 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine or red hot iron filings can render a medium reduced. Robertson cooked meat that is commonly used to grow Clostridium spps medium contain a 2.5 cm column of bullock heart meat and 15 ml of nutrient broth. Before use the medium must be boiled in water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin. Methylene blue or resazurin is an oxidation-reduction potential indicator that is incorporated in the thioglycollate medium. Under reduced condition, methylene blue is colourless.

Preparation and preservation

Care must be taken to adjust the pH of the medium before autoclaving. Various pH indicators that are in use include phenol red, neutral red, bromothymol blue, bromocresol purple etc. Dehydrated media are commercially available and must be reconstituted as per manufacturers' recommendation. Most culture media are sterililized by autoclaving. Certain media that contain heat labile components like glucose, antibiotics, urea, serum, blood are not autoclaved. These components are filtered and may be added separately after the medium is autoclaved. Certain highly selective media such as Wilson and Blair's medium and TCBS agar need not be sterilized. It is imperative that a representation from each lot be tested for performance and contamination before use. Once prepared, media may be held at 4-5^{o}C in the refrigerator for 1-2 weeks. Certain liquid media in screw capped bottles or tubes or cotton plugged can be held at room temperature for weeks.

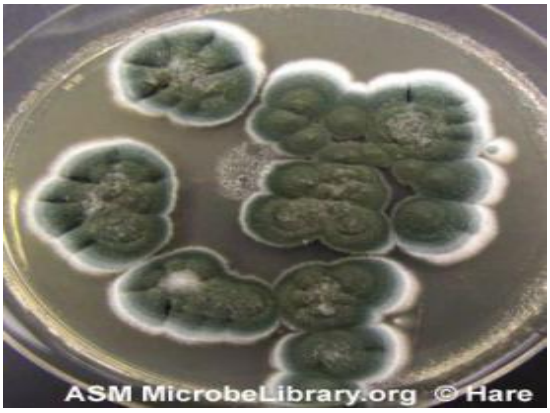
Types of fungal growth media

For optimal recovery of fungal pathogen, a battery of media should be used, and the followings are recommended:

1. Media with or without cyclohexamide (Cycloheximide is added to inhibit the growth of rapidly growing contaminating molds.)
2. Media with or without an antibacterial agent ((Chloramphenicol, Gentamicin and Ciprofloxacin are commonly used antibacterial for this purpose).

Antibacterial agents are used to kill the contaminating bacterial species. If the sample is taken from sterile site, it is not necessary to use media containing antibacterial agents.

1. **Brain-heart infusion (BHI) agar**: It is a nonselective fungal culture medium that permits the growth of virtually all clinically relevant fungi. It is used for the primary recovery of saprophytic and dimorphic fungi
2. **Czapek's agar**: It is used for the subculture of *Aspergillus* species for their differential diagnosis.
3. **Inhibitory mold agar (IMA):** Primary recovery of dimorphic pathogenic fungi. Saprophytic fungi and dermatophytes will not be recovered.
4. **Mycosel/Mycobiotic agar:**
    1. It is generally Sabouraud's dextrose agar with cycloheximide and chloramphenicol added.
    2. It is used for the primary recovery of dermatophytes.
    3. Niger Seed Agar: It is used for the identification of *Cryptococcus neoformans*.
5. **Potato Dextrose Agar (PDA):** It is a relatively rich medium for growing a wide range of fungi.
6. **Sabouraud's Heart Infusion (SABHI) agar:** Primary recovery of saprophytic and dimorphic fungi, particularly fastidious strains.



Penicillium notatum on Sabouraud agar
Image source: ASM

7. **Sabouraud's dextrose agar (SDA):**
    1. Sabouraud's agar is sufficient for the recovery of dermatophytes from cutaneous samples and yeasts from vaginal cultures.
    2. Not recommended as a primary isolation medium because it is insufficiently rich to recover certain fastidious pathogenic species, particularly most of the dimorphic fungi.
    3. Sabouraud's dextrose agar (2%) is most useful as a medium for the subculture of fungi recovered on enriched medium to enhance typical sporulation and provide the more characteristic colony morphology.
8. **Potato flake agar:** Primary recovery of saprophytic and dimorphic fungi, particularly fastidious and slow growing strains.

Bacterial Growth Curve

Objectives:

1. To study the different phases of bacterial growth.
2. To plot standard growth curve of *Staphylococcus aureus.*
3. To determine the generation time of given bacteria.

Principle:

The increase in the cell size and cell mass during the development of an organism is termed as growth. It is the unique characteristics of all organisms. The organism must require certain basic parameters for their energy generation and cellular biosynthesis. The growth of the organism is affected by both physical and Nutritional factors. The physical factors include the pH, temperature, Osmotic pressure, Hydrostatic pressure, and Moisture content of the medium in which the organism is growing. The nutritional factors include the amount of Carbon, nitrogen, Sulphur, phosphorous, and other trace elements provided in the growth medium. Bacteria are unicellular (single cell) organisms. When the bacteria reach a certain size, they divide by binary fission, in which the one cell divides into two, two into four and continue the process in a geometric fashion. The bacterium is then known to be in an actively growing phase. To study the bacterial growth population, the viable cells of the bacterium should be inoculated on to the sterile broth and incubated under optimal growth conditions. The bacterium starts utilising the components of the media and it will increase in its size and cellular mass. The dynamics of the bacterial growth can be studied by plotting the cell growth (absorbance) versus the incubation time or log of cell number versus time. The curve thus obtained is a sigmoid curve and is known as a standard growth curve. The increase in the cell mass of the organism is measured by using the Spectrophotometer. The Spectrophotometer measures the turbidity or Optical density which is the measure of the amount of light absorbed by a bacterial suspension. The degree of turbidity in the broth culture is directly related to the number of microorganism present, either viable or dead cells, and is a convenient and rapid method of measuring cell growth rate of an organism. Thus the increasing the turbidity of the broth medium indicates increase of the microbial cell mass (Fig 1) .The amount of transmitted light through turbid broth decreases with subsequent increase in the absorbance value.

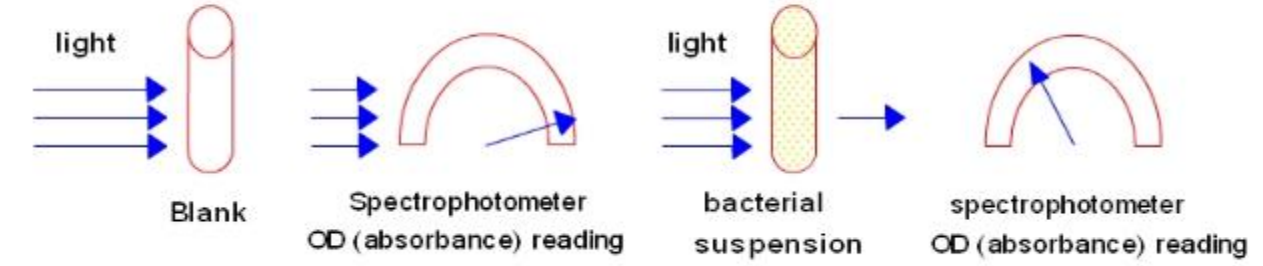


Fig 1: Absorbance reading of bacterial suspension

The growth curve has four distinct phases (Fig 2)

1. Lag phase

When a microorganism is introduced into the fresh medium, it takes some time to adjust with the new environment. This phase is termed as Lag phase, in which cellular metabolism is accelerated, cells are increasing in size, but the bacteria are not able to replicate and therefore no increase in cell mass. The length of the lag phase depends directly on the previous growth condition of the organism. When the microorganism growing in a rich medium is inoculated into nutritionally poor medium, the organism will take more time to adapt with the new environment. The organism will start synthesizing the necessary proteins, co-enzymes and vitamins needed for their growth and hence there will be a subsequent increase in the lag phase. Similarly when an organism from a nutritionally poor medium is added to a nutritionally rich medium, the organism can easily adapt to the environment, it can start the cell division without any delay, and therefore will have less lag phase it may be absent.

2. Exponential or Logarithmic (log) phase

During this phase, the microorganisms are in a rapidly growing and dividing state. Their metabolic activity increases and the organism begin the DNA replication by binary fission at a constant rate. The growth medium is exploited at the maximal rate, the culture reaches the maximum growth rate and the number of bacteria increases logarithmically (exponentially) and finally the single cell divide into two, which replicate into four, eight, sixteen, thirty two and so on (That is 2^0, 2^1, 2^2, 2^3.....2^n, n is the number of generations) This will result in a balanced growth. The time taken by the bacteria to double in number during a specified time period is known as the generation time. The generation time tends to vary with different organisms. *E.coli* divides in

every 20 minutes, hence its generation time is 20 minutes, and for *Staphylococcus aureus* it is 30 minutes.

3. Stationary phase

As the bacterial population continues to grow, all the nutrients in the growth medium are used up by the microorganism for their rapid multiplication. This result in the accumulation of waste materials, toxic metabolites and inhibitory compounds such as antibiotics in the medium. This shifts the conditions of the medium such as pH and temperature, thereby creating an unfavourable environment for the bacterial growth. The reproduction rate will slow down, the cells undergoing division is equal to the number of cell death, and finally bacterium stops its division completely. The cell number is not increased and thus the growth rate is stabilized. If a cell taken from the stationary phase is introduced into a fresh medium, the cell can easily move on the exponential phase and is able to perform its metabolic activities as usual.

4. Decline or Death phase

The depletion of nutrients and the subsequent accumulation of metabolic waste products and other toxic materials in the media will facilitates the bacterium to move on to the Death phase. During this, the bacterium completely loses its ability to reproduce. Individual bacteria begin to die due to the unfavourable conditions and the death is rapid and at uniform rate. The number of dead cells exceeds the number of live cells. Some organisms which can resist this condition can survive in the environment by producing endospores.

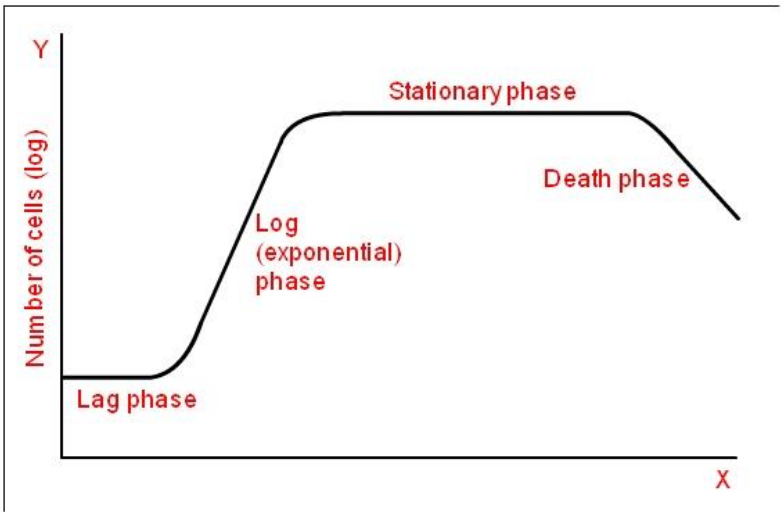


Fig 2: Different phases of growth of a bacteria

CALCULATION:

The generation time can be calculated from the growth curve(Fig 3).

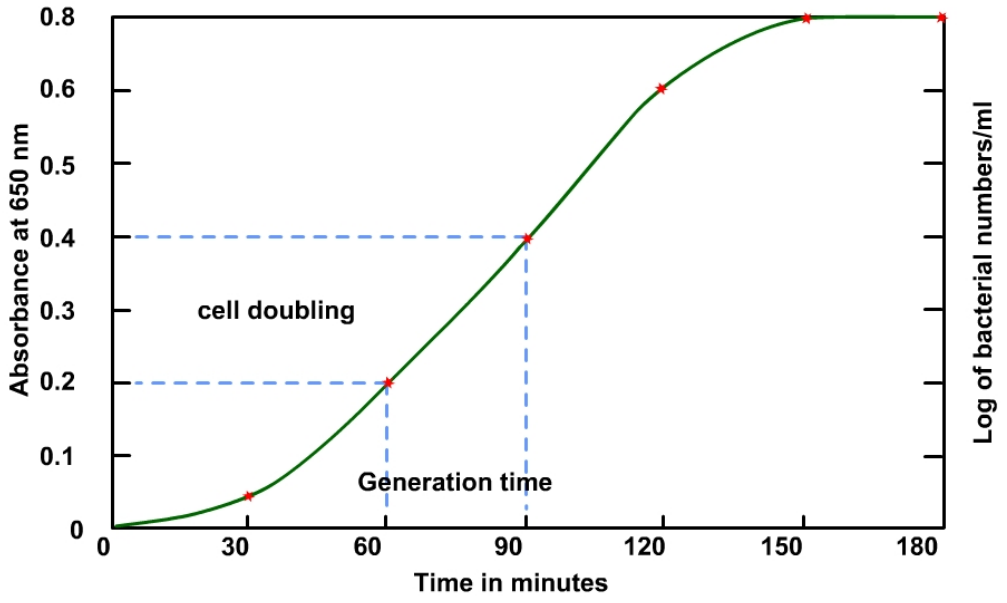


Fig 3: Calculation of generation time

The exactly doubled points from the absorbance readings were taken and, the points were extrapolated to meet the respective time axis.

Generation Time = (Time in minutes to obtain the absorbance 0.4) – (Time in minutes to obtain the absorbance 0.2)

= 90-60

= 30 minutes

Let No = the initial population number

Nt = population at time t

N = the number of generations in time t

Therefore,

$$Nt = No \times 2^n \ldots\ldots\ldots (1)$$

$$\log Nt = \log No + n\log 2$$

Therefore,

$$n = (\log Nt - \log No) / \log 2$$

$$n = (\log Nt - \log No) / 0.301 \ldots\ldots\ldots\ldots (2)$$

The growth rate can be expressed in terms of mean growth rate constant (k), the number of generations per unit time.

$$k = n / t$$

$$k = (\log Nt - \log No) / (0.301 \times t) \ldots\ldots\ldots\ldots\ldots (3)$$

Mean generation time or mean doubling time (g), is the time taken to double its size.

Therefore,

$$Nt = 2No \ldots\ldots\ldots\ldots\ldots (4)$$

Substituting equation 4 in equation 3

$$k = (\log Nt - \log No) / (0.301 \times t)$$
$$= (\log 2N0 - \log No) / (0.301 \times t)$$
$$= \log 2 + (\log No - \log No) / 0.301 \; g$$

(Since the population doubles t= g)

Therefore,

$$k = 1 / g$$

Mean growth rate constant, $k = 1 / g$

Mean generation time, $g = 1 / k$

Microbial Types based on Nutrition

Introduction

All those events through which necessary and directly usable supplies are delivered to and into individual cells comprise **nutrition**; and any chemical an organism requires as raw material may be called a **nutrient**. Since *a*living body consists partly of inorganic and partly of organic chemicals and since all life functions are directed towards maintaining and perpetuating the body, it is clear that an organism must supply itself with both inorganic and organic metabolites; we recall that organic metabolites are also called **food**.

The physical environment is the ultimate source, which supplies organisms with all required inorganic metabolites. From some of these, the required organic metabolites must then be manufactured and distributed within the living body itself. Thus all organisms build up their bodies at the ultimate expense of inorganic materials withdrawn directly from the physical environment. Excretion products formed within organisms return to the environment largely while the organisms live. And when they die, all other materials of their bodies return to the environment as well. As we shall see, decay caused by saprotrophic bacteria and fungi gradually retransforms all the returned substances into the same kinds of inorganic materials, which were withdrawn from the environment originally.

The global environment consists of three main subdivisions. The **hydrosphere** includes all liquid components i.e. the water in oceans, lakes, rivers and on land. The **lithosphere** comprises the solid components i.e. the rocky substance of the continents. And the **atmosphere**, which is the gaseous, mantle which envelopes the hydrosphere and lithosphere. Living organisms in general require inorganic metabolites from each of these subdivisions. The hydrosphere supplies liquid water; the lithosphere supplies all other minerals and the atmosphere supplies oxygen, nitrogen and carbon dioxide. Together these inorganic materials provide all the chemical elements needed in the construction and maintenance of living matter. In addition to being sources of supply the three subdivisions of the environment also affect metabolism in various other specific ways.

We shall examine in brief the nutritional aspects of organisms under the following broad heads; nutritional requirements and forms of nutrition

Nutritional Requirements

The microbial cells are extremely complex and in addition to oxygen and hydrogen they contain four other major elements such as carbon, nitrogen, phosphorus and sulphur. About 95 per cent of cellular dry weight in these organisms is accounted for these six elements. The microorganisms in general do not need only these six elements but also others, which are found in lesser -quantity. Such elements are potassium, magnesium, calcium, sodium, iron, manganese, cobalt copper, molybdenum and zinc. These elements, infact, are needed for survival and growth of the organisms. The microorganisms differ greatly with respect to the chemical form in which these elements are utilized as nutrients. This, however, holds good in case of carbon, nitrogen, sulphur and oxygen and therefore, the requirements for these four elements cannot be easily described. Carbon assumes great importance as the main constituent of all organic cell material and represents about 50 per cent of the cells dry weight. Nitrogen is found mostly in proteins, nucleic acids, co-enzymes etc. Phosphorus is a major constituent of nucleic acids while sulphur happens to be a constituent of mainly proteins and coenzymes.

Carbon dioxide is the most oxidized form of carbon and the photosynthetic microorganisms reduce CO_2 to organic cell constituents. On the other hand, all the non-photosynthetic microorganisms obtain their carbon requirement mainly from organic nutrients, which contain reduced carbon compounds. These organic compound not only-provide the carbon for synthesis but also meet the energy requirement by entering into energy yielding metabolic pathways and are eventually oxidized to CO_2. Some microbes have the ability to synthesize all their cellular components using a single organic carbon source while others in addition to this one major carbon source also need other complex carbon containing components, which they can not synthesize. These are called growth factors, which include vitamins. Some microbes can utilize more than one carbon compound and exhibit a great degree of versatility. The others, however, are specialized in this regard.

Sulphur and nitrogen are taken up by most organisms and are subsequently reduced within the cell and utilized in other biosynthetic processes. The sulphur and nitrogen requirements of most organisms can also be met with organic nutrients that contain these two elements in reduced organic combinations such as amino acids. A few microorganisms are capable of reducing elemental nitrogen to ammonia and this process of nitrogen assimilation is known as biological nitrogen fixation.

Most of the microorganisms need molecular oxygen for respiration. In these, the oxygen serves as terminal electron acceptor and, such organisms are referred to as '**obligate aerobes'**. As opposed to this there are a few organisms, which do not use molecular oxygen as terminal electron acceptor. We recall that oxygen is a component of the cellular material of all the microorganisms. These microbes are called '**obligate anaerobes'**. In fact, molecular oxygen in toxic to these organisms. Aerobes, which can grow in the absence of oxygen, are called '**facultative anaerobes'** and the anaerobes which can grow in the presence of oxygen are referred to as '**facultative aerobes'**. In addition to these major classes, there are organisms, which grow best at reduced oxygen pressure but are obligate aerobes and these are called '**Microaerophilic'**.

In addition to the four major elements (CNPS) the microorganisms also need a large number of metals in trace quantities. These trace elements are known to be essential for functioning of various enzymes in the microbial cells. Apart from the trace elements the microbes may also need some growth factors (vitamins) which can not be synthesized from single carbon or nitrogen sources and which must be supplied to these organisms to allow their proper growth and development.

The Nutritional Forms

Various nutritional patterns .may be distinguished on the basis of how microorganisms obtain their required organic and inorganic metabolites. In fact, microorganisms do not differ with respect to their procurement of inorganic metabolites; all microorganisms obtain them in finished, prefabricated form from their environment. However, microorganisms do differ with respect to their procurement of organic metabolites (foods). Some microorganisms manufacture their foods from inorganic supplies to them and thus are able to subsist in an exclusively inorganic environment: They are collectively called **autotrophs**. Other micro organic metabolites; they must absorb from the environment certain minimum amounts and kinds of prefabricated organic metabolites (the foods). Such microorganisms are collectively called **heterotrophs**.

Autotrophic microorganisms, which manufacture foods from inorganic sources, require not only external source of appropriate nutrient raw materials but also external sources of energy. In some cases, external energy for food manufacture is obtained from light and such microorganisms are collectively called **photosynthesizers**. In other cases, some inorganic nutrients serve as raw materials for food manufacture

and other inorganic nutrients, i.e., chemicals, serve as external energy sources. Such microorganisms are collectively called**chemosynthesizers**.

We may, therefore, characterize nutritional forms from the standpoint of nature of food procurement, i.e., its autotrophic (manufacture) or heterotrophic (absorptive) nature, and we may also characterize it from the standpoint of external energy sources, i.e., its photosynthetic or chemosynthetic nature. If we characterize it from both standpoints simultaneously, we may identify following four categories of microorganisms:

Photoautotrophs

Which use light as energy Source and manufacture their food from inorganic raw materials.

Chemoautotrophs

Which use chemicals (inorganic) as energy source and manufacture their food from inorganic raw materials.

Photoheterotrophs

Which use light as energy source and convert reduced organic materials into usable foods.

Chemoheterotrophs

Which use chemical (organic) as energy source as well as absorb some organic chemicals as direct food.

Contrary to the above, if we emphasize raw material sources (i.e., inorganic or organic) and sources of energy (i.e., light or chemicals) simultaneously, we may categorize microorganisms into following four categories:

Photolithotrophs

Which use light as energy source and inorganic materials to obtain their food.

Chemolithotrophs

Which use chemicals as energy source and inorganic materials to obtain their food.

Photoorganotrophs

Which are light as energy source and reduced organic materials to obtain their food.

Chemoorganotrophs

Which use organic chemicals as energy source as well as direct food.

In both type of categories of nutritional forms the first two categories include all the autotrophic microorganisms; the last two, all the heterotrophic microorganisms. The first and third categories include all the

photosynthetic micro-organisms; the second and forth, all the chemosynthetic microorganisms (Fig.1.)

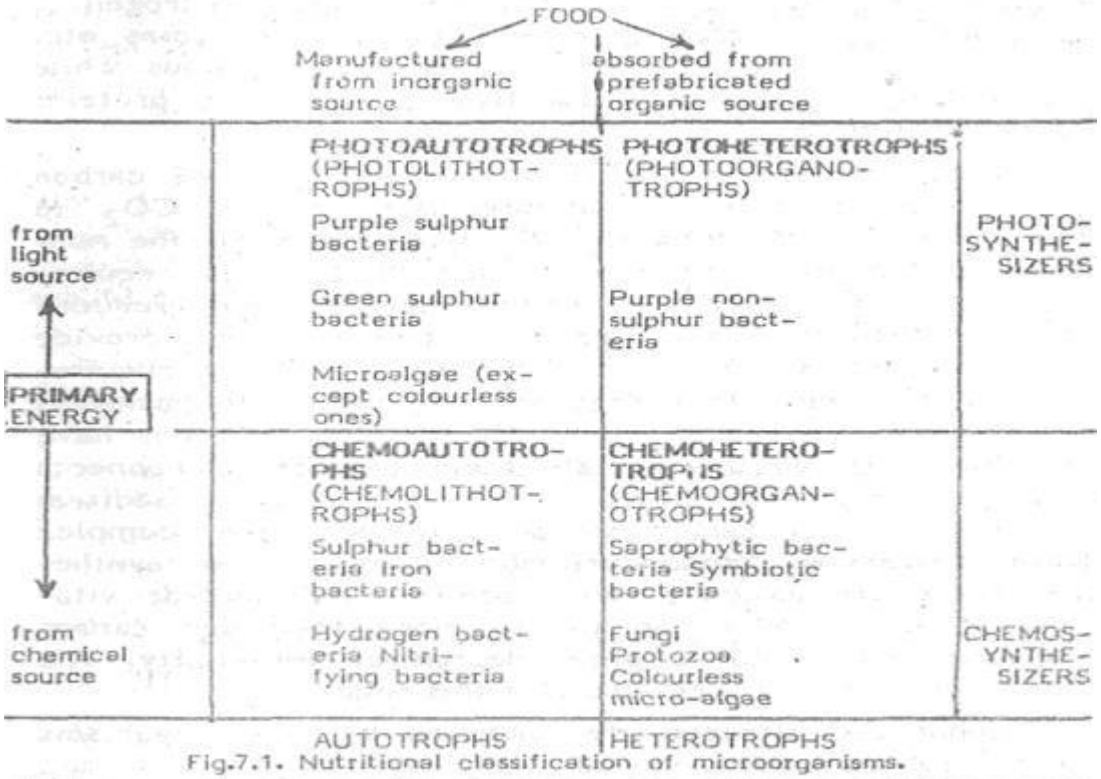


Fig.7.1. Nutritional classification of microorganisms.

* These are the intermediate forms between autotrophs and heterotrophs; sometimes they behave as photoautotrophs and at others as chemoheterotrophs.

Fig.1 Nutritional Classification of Microorganisms

Autotrophic Microorganisms

In as much as the simplest organic metabolites, i.e., carbohydrates, contain the elements carbon, hydrogen and oxygen, it is clear that every autotrophic microorganism requires an inorganic source of each of these elements. In addition, an external energy source is required to combine the three elements into organic substances.

Autotrophic microorganisms of all kinds use environmental carbon dioxide as their inorganic carbon and oxygen source.

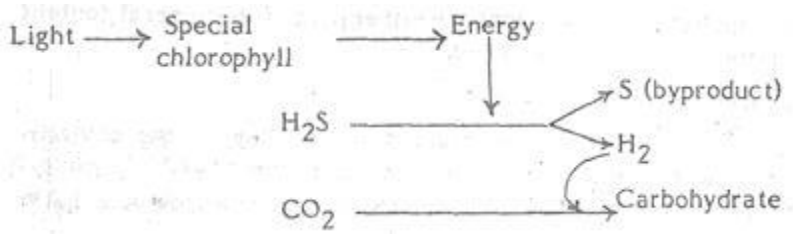
In virtually all cases, the carbohydrate formed is a derivative of glucose, and from this basic food all the other constituents of the microorganism are then produced. But, however, autotrophic microorganisms differ in their energy and hydrogen sources; and taking this aspect into account they can be classified into following two categories:

2.1.1. Photoautotrophs

As in the Fig.1 indicates, this group includes most pigmented bacteria and microalgae (other than colourless ones). In all these photosynthetic microorganisms, the external energy source is **light**, and one or more varieties of**chlorophyll** are present to trap the energy of light. Such microorganisms are therefore largely green, but in many cases other pigments obscure or completely mask the colour of chlorophyll.

The hydrogen source of all photoautotrophic microorganisms except the photoautotrophic bacteria is**environmental water**. Light energy is used to split water and free hydrogen is made available.

The hydrogen source of photoautotrophic bacteria is not water and oxygen is never a byproduct of photo-synthesis. These bacteria are adapted to live in sulphur springs and other sulphurous regions, where hydrogen sulphide (H_2S) is normally available. This compound generally serves as the hydrogen source. Two families of bacteria belong to photoautotrophic group, the purple sulphur bacteria and the green sulphur bacteria. The former possess a variety of special chlorophyll known as bacteriochlorophyll. It is green but its colour is masked by the additional yellow carotenoids, which are also present. Green sulphur bacteria possess **chlorobium-chlorophyll**or **bacteriviridin**, which is different from bacteriochlorophyll. Also, its green colour is not masked by the additional yellow carotenoids. For the photoautotrophic bacteria, therefore, the special-pattern of photosynthesis becomes:



Elemental sulphur is the byproduct. It is stored inside the cells in the purple sulphur bacteria, and it is excreted from the cells in the green sulphur bacteria.

2.1.2. Chemoautotrophs

This second group of autotrophic microorganisms consists entirely of bacteria. They can not use light, and their external energy sources in food manufacture are a variety of inorganic metabolites absorbed from the environment. In most cases, these metabolites are combined with oxygen in the cells, resulting in energy and a variety of inorganic byproducts. Water and carbon dioxide are the inorganic raw materials in subsequent food manufacture.

Among the best known chemoautotrophic microorganisms are the **sulphur bacteria**, the **iron bacteria**, the**nitri-fying bacteria** and the **hydrogen bacteria**.

(i) Sulphur bacteria

These bacteria absorb either hydrogen sulphide (H_2S) or molecular sulphur (S_2) from the environment and combine these metabolites with molecular oxygen. The resulting energy is used toward food manufacture and the byproducts are either S_2 (if H_2S is original nutrient) or sulphate ions (SO_4, if S_2 is the original nutrient):

If the byproduct is sulphur, granules of these elements are deposited inside or outside the cell; if the

byproduct is sulphate, these ions are either part of the mineral content of the cell or are excreted.

(ii) Iron bacteria

These bacteria are stalked ones living in the environment where iron compounds combine them with molecular oxygen, thereby converting them into insoluble substances, energy is gained in the process:

Soluble Compounds Fe Insoluble Fe Compounds

Energy

(iii) Nitrifying bacteria

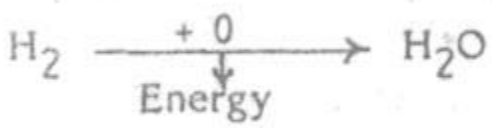
These bacteria are of two types, one using ammonia and excreting nitrite ions, the other using nitrite ions and excreting nitrate ions. Both types combine their specific nutrient with molecular oxygen and in each case energy is gained:

For the bacteria themselves, the important product here is energy, which makes food manufacture possible. For all other living organisms, the important products are the excreted byproducts, which make the global nitrogen

cycle possible.

(iv) Hydrogen bacteria

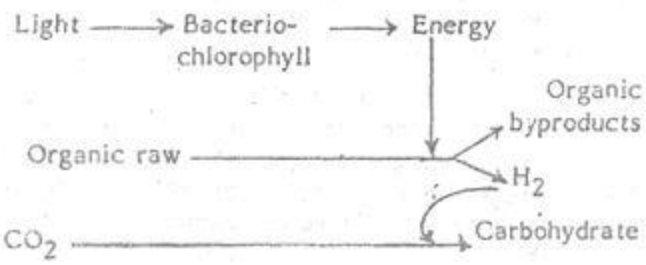
These bacteria utilize molecular hydrogen as nutrient. By combining such hydrogen with oxygen, energy is gained and water forms as a byproduct.



Intermediate Forms: Photoheterotrophs

The identifying feature of this interesting nutritional form is that the external energy source is **light** (i.e., the microorganisms are photosynthesizers), but, they require organic raw materials nevertheless. This peculiar form of nutrition, namely, photoheterotrophy, is absolutely unique to one small group of photosynthetic bacteria, the **purple nonsulphur** bacteria. Like the purple sulphur bacteria, the purple nonsulphur bacteria possess **bacteriochlorophyll** as well as red and yellow corotenoids, which mask the colour of the green pigment.

Purple non-sulphur bacteria absorb organic raw materials from the environment, but these metabolites are not or can not be used directly as foods. Instead, the organic materials serve as sources of hydrogen. Extracted hydrogen is then combined with carbon dioxide. CO_2 is usually produced in the oxidation of the organic compound and at the same time used as the hydrogen acceptor. The resulting carbohydrates do serve as usuable foods:



Purple nonsulphur bacteria may manufacture foods in this manner only if light is present and oxygen is**absent**. In some species, a different type of nutrition may also occur in the dark and in the presence of oxygen. Under such conditions, absorbed organic raw materials may be used as foods **directly**. In other words, these microorganisms then are "Chemoheterotrophs". If free H_2(g) is available, the Photoheterotrophs can use it to reduce CO_2 to carbohydrate (CH_2O) at the expense of light energy and they can exist as "Photoautotrophs".

Heterotrophic Microorganisms

Heterotrophic microorganisms can acquire inorganic metabolites such as H_2O and CO_2 from the environment but can not convert them into foods. They must, therefore, absorb prefabricated organic raw materials from the environment and use them directly as food. It follows that the survival of heterotrophic microorganisms is strictly contingent on the preexistance of autotrophs, for these must be the ultimate sources of the needed organic metabolites.

Chemoheterotrophs:

Majority of heterotrophic microorganisms belongs to this nutritional category, in which organic raw materials represent prefabricated, directly usable foods. Chemoheterotrophs can only use foods, not produce them.

Three kinds of microorganisms are included in the group: Holotrophs, Saprotrophs and Symbionts of various kinds.

i. Holotrophs

Holotrophs are the free-living bulk-feeders, i.e. most colourless Protista. All have mouth, if not, equivalent ingesting structures, and they digest ingested food and egest any unusable remains.

ii. Saprotrophs

Saprotrophs comprise the slime molds, most colourless algae, and, above all, very many bacteria and fungi. They all subsist on dead organisms or on nonliving derivatives of organisms: dead plants and animals of all kinds; dung, sewage and other elimination and excretion products; and derived materials such as milk, bread and leather. In short, anything and everything nonliving that contains organic components is likely to provide usable food for Saprotrophs. These microorganisms decompose such organic matter chemically and absorb nutrient molecules from the resulting juices. Thus, Saprotrophs bring about **decay**. It is important to note that decay occurs only if

and when Saprotrophs are at work.

As a result of their decay-causing nutritional activities, Saprotrophs are vital links in global nutrient cycles. The final decomposition products of decaying organic matter are H_2O, CO_2, and N_2, and these materials return to the environment from which living matter obtained them originally. Ail the decay-causing microorganisms which participate in the water, oxygen and carbon cycles belong to the saprotrophic Chemoorganotrophs; and those which participate in the nitrogen cycle, three are likewise saprotrophic: the decay causing bacteria and fungi, some of the denitrifying bacteria, and some of the nitrogen-fixing bacteria.

iii. Symbionts

Symbionts include commensalistic, mutualistic Helotic and parasitic types of microorganisms. Most of the microorganisms within each of these categories must absorb inorganic and organic nutrients in pre-fabricated molecular form directly from their hosts. Specific food requirements vary greatly. For example, one bacterial parasite may have to obtain a given vitamin or amino acid in prefabricated form, but another may be able to manufacture such a nutrient from other organic starting materials. Biochemical differences of this sort are exceedingly numerous, and they are one reason why a symbiont can not pick hosts at random. Survival is possible only in hosts in which all required types of nutrients are available.

Physical Conditions Required For Growth

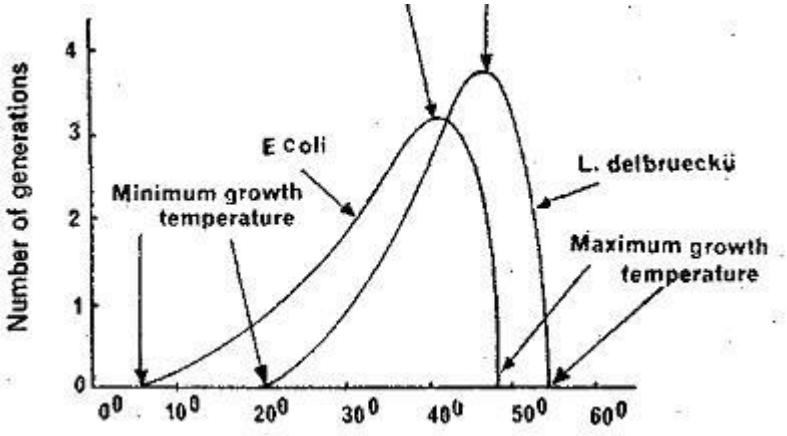
To cultivate microorganisms in the laboratory, it is not enough to determine their nutritional requirements, but also the physical environment where the organisms will grow best. Just as microorganisms vary greatly with regard to their nutritional requirements, so also they exhibit diverse response to the physical conditions in their environment. Some important physical factors that affect the growth of microorganisms are discussed.

1. Temperature

Temperature is the most important factor that determines the rates o£ growth, multiplication, survival, and death of all living organisms. Growth and reproduction of living organisms are dependent on a co-ordinated series of enzyme-catalysed chemical reactions. The rates of enzyme reaction increase with the increase in temperature. Since micro-bial activity and growth are manifestations of enzymatic reactions, their rates of growth are, temperature-dependent. In short.temperature determines the rate of growth, the total amount of growth, the metabolic activity, and the morphology of the organisms. Each microorganism can grow only within a growth temperature range characteristic of the species. The temperature relationships of a microorganism are usually described by the three cardinal temperatures, the *minimum, optimum,* and the*maximum temperatures* of growth.

The lowest temperature at which organisms grow is the *minimum growth temperature.* Most organisms will survive for a varying length of time below this temperature, but will show negligible growth. Minimum growth temperature is difficult to determine precisely.because of an increase in generation time. Growth is not visible until a population of about 1×10^7 cells /ml has been attained. Whether an organism is capable of growth at a particular temperature depends on the visibility of the growth.The *maximum growth temperature* is the highest temperature at which growth occurs. A temperature only slightly above this point frequently kills the microorganisms by inactivating critical enzymes. Maximum growth temperature is relatively easy to establish, because organisms either grow or are destroyed by high temperature. The *optimum temperature* is commonly

defined as the temperature at which the most rapid rate of multiplication occurs.For most organisms, optimum growth occurs over a temperature range rather than at a fixed temperature. The optimum temperature is also difficult to agree ,upon, for the optimum temperature of growth may not be optimum for other cellular activities, for example, maximum acid production or pigment production, Sometimes it also changes the nutritional requirement. Generally the upper limit of the optimum growth temperature is only a few degree below the maximum growth temperature. Fig. 17.11 illustrates the effect of temperature on the rate of two bacterial species.



Incubation temperature

Fig. Effect of temperature on the rate of logarithmic multiplication of of Escherichia coli and Lactobacillus delbrueckii

Maximum growth temperatures are only 5 to 10 degrees higher than the optimum growth temoeratures, whereas minimum growth temperatures are approximately 30 degrees lower.

Classification of bacteria according to growth temperature.

The numerical values of the *cardinal temperatures* (minimum, optimum and maximum), and the range of temperature over which growth is possible, vary widely among bacteria. Some bacteria isolated from hot springs are capable of growth at temperature as high as 95°C; others, isolated, from cold environments, can grow at temperature as low as - 10°C if a high solute concentration prevents the medium from freezing. Bacteria are frequently classified into three groups according to their temperature preferences. These groups are not sharply defined, and the distinctions are arbitary. However, this sort of classification is useful in describing the collective properties of groups of microorganisms adapted to life in certain environments. Bacteria are normally classified into three broad groups, *psychrophiles, mesophiles,* and *thermophiles.*

Psychrophiles. *Psychrophilic* (Or. *Psychros* = cold) bacteria are the predominant organisms in many uncultivated soils, and in lakes, streams, and oceans. They are commonly defined as micro-organisms capable of growth at 0°C, though they grow best at higher temperatures, between 15° to 30°C. Two groups of psychrophiles have been distinguished : (1) *obligate psychrophiles* cannot grow at temperatures above 19° to 22°C, whereas *(2) facultative psychrophiles* may grow at 30° to 35°C.

Mesophiles. Most of the commonly studied bacteria are m*esophilic* (Gr. *meso* = middle), and these fall into two

well defined sub-divisions: (1) those whose optimum growth temperatures are from 20° to 35°C., and (2) those whose optimum temperatures are between 35 to 45°C. The first group consists mainly of saprophytes and plant parasites, whereas the second group consists mainly of animal parasites or commensals. Minimum and maximum growth temperatures, vary correspondingly, but for the most part are within the range of 10 to 52°C.

Thermophiles. *Thermophiles* (Gr. Thermo=heat) have optimum growth temperature of 45°C or higher, and generally grow over a range of 40 to 75°C. Two groups of thermophiles have been observed. *Obligate thermophiles* grow only at high temperatures, usually above 50°C. *Facultative thermophiles* grow both at 37°C and 55°C. An organism that is beat resistant, for instance, one that withstands pasteurization, but does riot grow at high temperatures, is termed *thermoduric*.

Table. Classification, of bacteria according to their growth temperature.

Group	Temperature range	Sub-division
1. Psychrophiles	Grow Well at O'C. (a) cannot grow at temp, above 19° to 22'C.	Obligate psychrophiles.
	(b) may grow at 30° to 35'C	Facultative psychrophiles.
2. Mesophiles	Do not grow at 0^8C. Optimum growth temperature is less than	
	45°C.	
	(a) optimum growth temp. 20°	Saprophytes.
	to 35'C.	
	(b) optimum growth temp. 35*C	Animal
	to 45^8C.	parasites.
3. Thermophiles	Optimum growth temperature is greater than 45°C.	Obligate ther-
	(a) grow above 50"C.	mophiles.
	(b) grow both at 37°C and above	Facultative
	50*C.	thermophiles.

2. Gaseous requirements.

The principal gases that affect microbial growth are oxygen and carbon dioxide. The present atmosphere of the earth 6oniains about 20 percent (V/V) oxygen. Although almost all higher plants and animals are dependent upon a supply of

oxygen, this does not hold true for all microorganisms. The responses to oxygen among microorganisms are remarkably variable, and this is an important factor in their cultivation. The organisms are divided into four groups on the basis of their relationship to molecular oxygen :

i. Strict or obligate aerobex grow only in the presence of free oxygen.

ii. Strict or obligate anaerobes grow only in the absence of free oxygen.

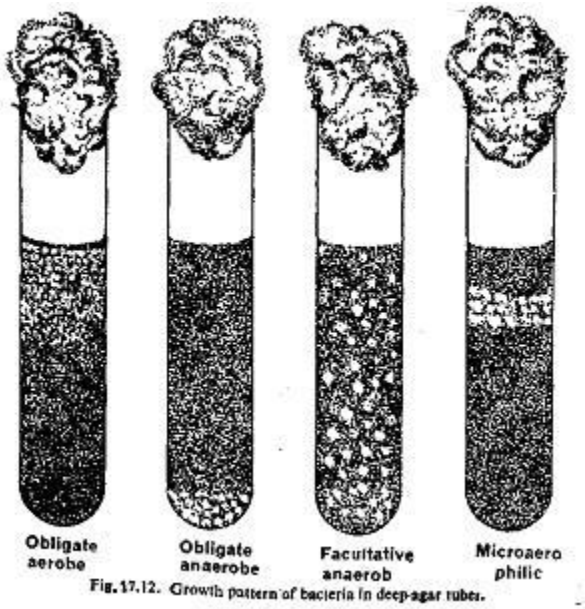


Fig. 17.12. Growth pattern of bacteria in deep-agar tubes.

iii. *Facultative anaerobe* can grow both in the presence and the absence of free oxygen

iv. *Mlcroaerophilic* organisms grow best in the presence of a low concentration of molecular oxygen.

Oxygen requirements. Molecular oxygen is relatively insoluble in water, and so must be continuously made available to aerobic microorganisms. Growth of aerobic microorganisms in tubes or small flasks incubated under normal atmospheric conditions is generally satisfactory. However, when aerobic organisms are to be grown in large quantities, it is advantageous to increase the exposure of the medium to the atmosphere. This can be accomplished by dispensing the medium in shallow layers, for which suitable containers are available. Alternatively, shaking and bubbling in sterile air or oxygen is done for increasing the availibility of oxygen to microorganisms growing in a liquid medium. However, the amount of oxygen required by various aerobic microorganisms differs considerably. Also, the amount of oxygen required for maximum growth can differ from that required for other metabolic processes. For example, the amount of oxygen required for the growth of*Aspergilus niger* is less than that required for the production of citric acid by *A. niger.*

To cultivate anaerobic microorganism, special techniques are devised to exclude all atmospheric oxygen from the medium. Anaerobic environment can be established by using one of the following methods.

i. Addition of reducing compounds, e.g. sodium thioglycollate, cysteine hydrochloride, sodium formaldehyde,

sulphoxalate, etc. to the medium to absorb oxygen

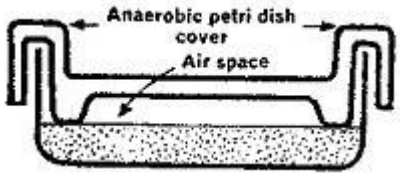


Fig. Anaerobic plate cultivation. Cross section showing Brewer anaerobic petrj dish cover in use. The anaerobic agar contains the reducing agent, sodium thioglycollate. Note that at the periphery of the agar surface the petri dish cover js in contact with the agar, thus sealing the air space.The thioglycollate absorbs the oxygen from the air space.

ii. Mechanical removal of oxygen from an enclosed vessel containing tubes or plates of inoculated medium. The air is pumped out of the vessel and replaced by nitrogen, helium, or a mixture of nitrogen and carbon dioxide

i. iii. Chemical reaction within an enclosed vessel containing the incubated medium, to combine the free oxygen into a compound. This can be as simple as the bu rning of a small candle or the combustion of small amount of alcohol to use up some of the free oxygen. A common laboratory method of cultivating an anaerobic micro organism by introducing pyragallol over the cotton plug in the inoculated slant tube is illustrated in Fig.

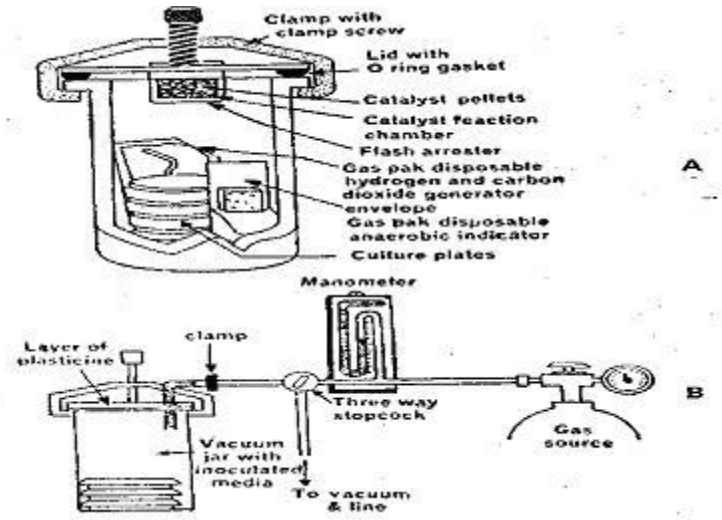


Fig. The Gaspak anaerobic system

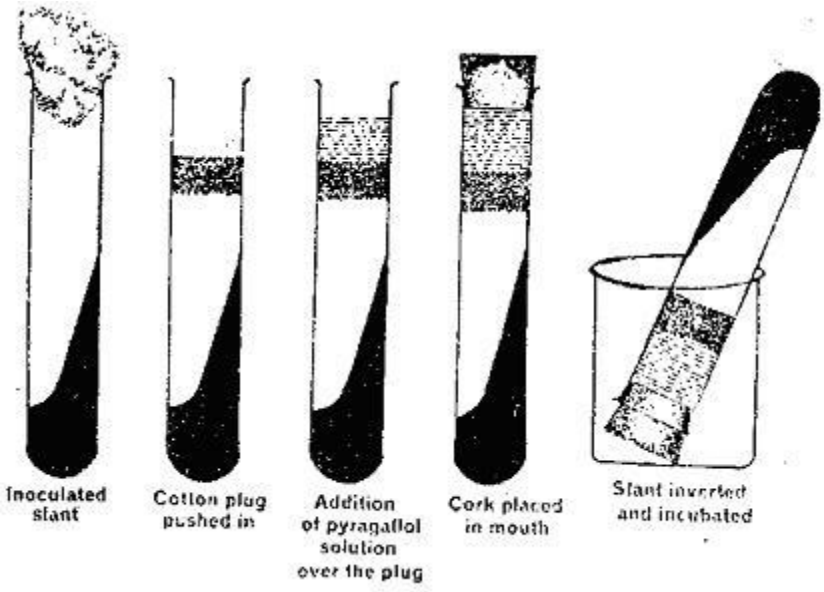


Fig. Anaerobic slant cultivation

Carbon dioxide requirement. All microorganisms utilize carbon dioxide for growth. In some microorganisms the liberation of carbon dioxide from metabolic reactions is adequate to supply this need. However, when cultures are vigorously aerated, particularly when there is a low cell density, the air may sweep the CO_2 away as quickly as it is produced. Secondly, a sufficient amount of carbon dioxide is to be provided for the cultivation of*autotrophx*. In case of autotrophs that can be grown under anaerobic condition, the requirement of CO_3 can be met by providing buffers such as $CaCO_3$ or $NaHCO_3$ which release $C0_2$ when acid is produced by the culture. Carbonates cannot be used in media exposed to air, because the release of CO2 is rapidly swept away, causing the medium to become extremely alkaline.

3. Hydrogen ion concentration (pH).

Small size and great mobility of hydrogen ions are of supreme importance in many chemical processes, and more so in biological processes, because of the transfer of hydrogen from one molecule to another. The tendency of hydrogen to dissociate from its original combination thus determines the probability of the reaction. The concentration of hydrogen is always low in the natural habitat of micro-organisms, but on the other hand the organisms cannot grow in its complete absence. The effect of hydrogen ions is similar to that of metallic ions, high concentration is toxic, moderately low concentration permits growth, and very low concentration is unfavourable for growth.

The **acidity or alkalinity** of a solution is a function of the relative hydrogen ion (H+) concentration or pH which is expressed as the negative log of the hydrogen ion concentration. Microbial growth and activities are strongly affected by the pH of the medium. However, there are wide differences between the pH requirements of the various species. These differences reflect the normal habits and habitats of the organisms. Microorganisms show the same type of tolerance to acidity or alkalinity that was observed for temperature. Each species usually shows a range of growth responses to varying pH values., and have a pH optimum for maximal balanced growth. Organisms which require pH values of 5 or less for maximal growth rate are termed ***acidopliiles***, and usually have a pH optium of 2 or 3. ***Alkaliphiles*** grow at pH value between 7 and 12, with the optimum around pH

9.5.*Neutro-philes* prefer pH values around neutrality (pH 7).

Bacteria, in general, prefer media of pH values near neutrality, and usually cannot tolerate pH values much below 4-5. There arc some exceptions to this generalization. The classic example is *Thiobacillus thioxidans,* which oxidizes sulphur to sulphuric acid, can grow . at pH 1.0 Acetic acid bacteria and intestinal bacteria which tolerate the acid of the stomach are other exceptions. Animal pathogens are usually favoured by an environment at pH 7.2 to 7.4. At the opposite extreme, bacteria that infect the human urinary tract and hydrolyze urea to give ammonia can grow at pH 11. Yeasts prefer slightly acidic media for growth. Moulds prefer more acidic media (pH 4). Many plant and soil microorganisms, especially *Actinomycetes,* prefer relatively alkaline conditions.

When microorganisms are inoculated in a medium originally adjusted to a given pH, it is very likely that this pH will change, depending upon the type of the microbial activity and the composition of the medium. Degradation of proteins and other nitrogenous compounds frequently yields ammonia or other alkaline byproducts; carbohydrate fermentations often produce organic acids. The change in the pH value brought by such reactions continues until the maximum or minimum pH for the organisms is reached, whereupon the culture dies.

The pH of the medium also determines which pathways of metabolism will operate. For example, at an alkaline reaction yeasts ferment glucose to glycerol, whereas at an acid reaction they ferment glucose to ethanol- Organisms such as *Aerobacter aerogenes,* which can form acetylmethyl-carbinol from glucose, will do so only below pH 6.0. The fate of amino acids in the cell is also decided by pH. At an acid reaction they are decarboxylated to the corresponding amines, whereas at alkaline reaction they are deaminated to an acid.

Buffers are often added to prevent the radical shift in the pH of the medium. Most buffers used in media arc mixtures of weakly acidic and weakly alkaline compounds. A combination of KH_2PO_4 and $K2HPO_4$ is widely employed in bacteriological media. If microorganisms form an acid such as acetic acid in a medium buffered with phosphate, a part of the basic salt (K_2HPO_4) is converted to the weakly acidic salt.

$K_2HPO_4 + HC_2H_30_2 \leftarrow \rightarrow KH_2PO_4 + KC_2H_3O_2$

The pH of the medium falls only slightly. Conversely, a basic microbia! product reacts with the acidic salt (KH_2PO_4) to forma dibasic compound that is only weakly alkaline. Many culture media contain amphoteric substances such as peptones. These compounds possess both amino and carboxyl radicals, which can dissociate as basic and acidic groups- Insoluble carbonates such as $CaCO_3$ aud $MgCO_3$ are also added to media to prevent a drop in pH as acid is produced. Being insoluble, they have no direct effect on pH, but when acid is formed and the reaction falls below pH 7.0, the carbonate decomposes, CO_2 is evolved, and the acid is converted to its calcium or magnesium salt- 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.

4. Miscellaneous physical requirements. Additional physical factors are to be considered for the growth of certain fastidious organims. For example *photosynthetic* microorganisms(algae, photosynthetic bacteria) must be exposed to a source of illumination, since light is their source of energy. *Halopfules* and *osmophiies* isolated from sea and other natural bodies of water of high salinity can grow only when the medium contains an unusually high concentration of salt.The successful cultivation of microorganism in the laboratory is based upon two basic principles; nutritional requirement to prepare a suitable nutrient medium, and appropriate physical conditions to obtain maximum growth.

Classification of organisms based on their metabolism

Energy source	sunlight	photo-			-troph
	Preformed molecules	chemo-			
Electron donor	organic compound		organo-		
	inorganic compound		litho-		
Carbon source	organic compound			hetero-	
	carbon dioxide			auto-	

Requirements for Growth	**Form usually found in Nature**	**Chemical Form commonly added to Microbiological Media**
Carbon	Carbon dioxide (CO_2), HCO_3^- organic compounds	Organic; simple sugars e.g. glucose, acetate or pyruvate; extracts such as peptone, tryptone, yeast extract etc. Inorganic; carbon dioxide (CO_2) or hydrogen carbonate salts (HCO_3^-)*
Hydrogen	Water (H_2O) organic compounds	
Oxygen	Water (H_2O), oxygen gas (O_2), organic compounds	
Nitrogen	Ammonia (NH_3), nitrate (NO_3^-) organic compounds e.g. amino acids nitrogen gas (N_2)	Organic; amino acids, nitrogenous bases Inorganic; NH_4CI, $(NH_4)_2S0_4$, KNO_3, and for dinitrogen fixers N_2
Phosphorus	Phosphate (PO_4^{3-})	KH_2PO_4, Na_2HPO_4*
Sulphur	Hydrogen sulphide(H_2S), sulphate (SO_4^{2-}), organic compounds e.g cysteine	Na_2SO_4, H_2S
Potassium	K^+	KCI, K_2HPO_4*
Magnesium	Mg^{2+}	$MgCI_2$, $MgSO_4$
Calcium	Ca^{2+}	$CaCI_2$, $Ca(HC0_3)_2$*
Sodium	Na^+	NaCI
Iron	Fe^{3+} organic iron complexes	$FeCI_3$, $Fe(NH_4)(SO_4)_2$, Fe-chelates[1)]
Trace elements	Usually present at very low concentrations	$CoCI_2$, $ZnCI_2$, Na_2MoO_4, $CuCI_2$, $MnSO_4$, $NiCI_2$, Na_2SeO_4, Na_2WO_4, Na_2VO_4
Organic growth factors	Usually present at very low concentrations	Vitamins, amino acids, purines, pyrimidines

Factor	Class of Organism	Minimum	Optimum	Maximum	Example
Temperature (°C)	extreme psychrophile	-2	5	10	*Raphidonema nivale*
					(snow algae)
	psychrophile	0	15	20	*Vibrio marinus*
	mesophile	10-15	24-40	35-45	*Escherichia* coli
	facultative thermophile	37	45-55	70	*Bacillus stearothermophilus*
	obligate thermophile	45	70-75	85-90	*Thermus aquaticus*
	extreme thermophile	60	75-80	85-110	*Sulfolobus acidocaldarius*
Oxygen Requirements	Aerobes				*Escherichia* coli
	Anaerobes				*Clostridium butulinum*
	Microaerophiles				
pH	acidophile	0.8	2-3	5	*Thiobacillus thiooxidans*
	alkal(in)ophile	ca 7	9-10.5	11-11.5	*Bacillus alcalophilus*
Osmotic pressure	halophile	0.5	1-2	4-4.5	*Vibrio costicola*
(Molar salt conc)	extreme halophile	3	3 5	5.2	*Halobacterium halobium*

Pure culture techniques

Obtaining Pure Cultures

- Pure culture: a culture that contains only a single species or strain of organism
- A colony is a population of cells arising from a single cell or spore or from a group of attached cells
- A colony is often called a colony-forming unit (CFU)
- The streak plate method is used to isolate pure cultures

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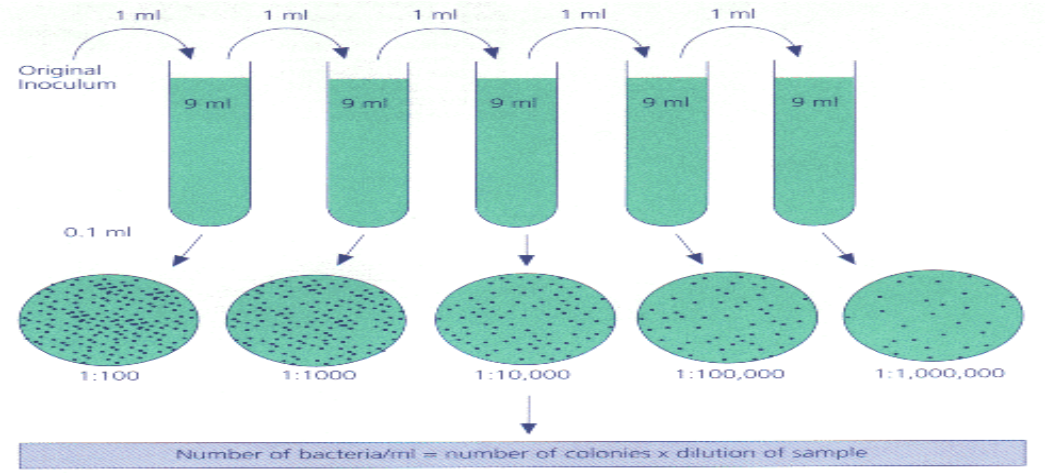
Types

1. Serial dilution method

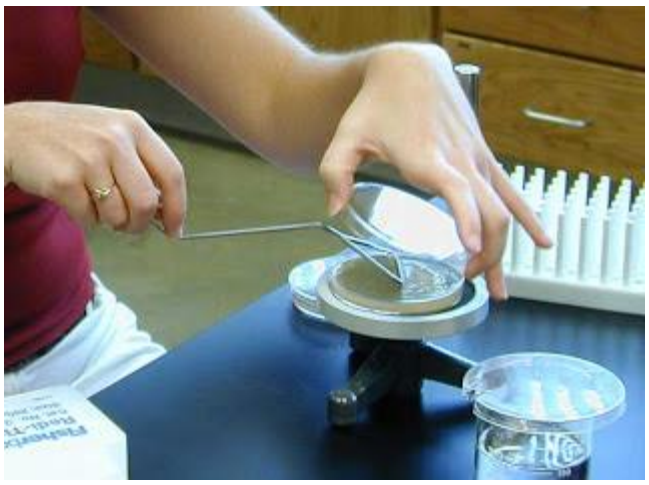


Serial Dilutions

The inoculum is diluted out in a series of dilution tubes which are plated out. The number of colonies on the plate are counted and corrected for the dilution to calculate the number of organisms in the original inoculum.

2. Spread plate method

Spread plate technique is an additional method of quantifying microorganisms on solid medium. With the spread plate method, a volume of an appropriately diluted culture usually no greater than 0.1 ml is spread over the surface of an agar plate using a sterile glass spreader. The plate is than incubated until the colonies appear, and the number of colonies counted. Instead of embedding microorganisms into agar, as is done with the pour plate method, liquid cultures are spread on the agar surface.



An advantage of spreading a plate over the pour plate method is that cultures are never exposed to 45 ^{o}C (i.e. melted agar temperatures).
Note: Surface of the plate must be dry, so that the liquid that is spread soaks in. volume greater than 0.1ml are rarely used because the excess liquid does not soak in and may cause the colonies to coalesce as they from, making them difficult to count.

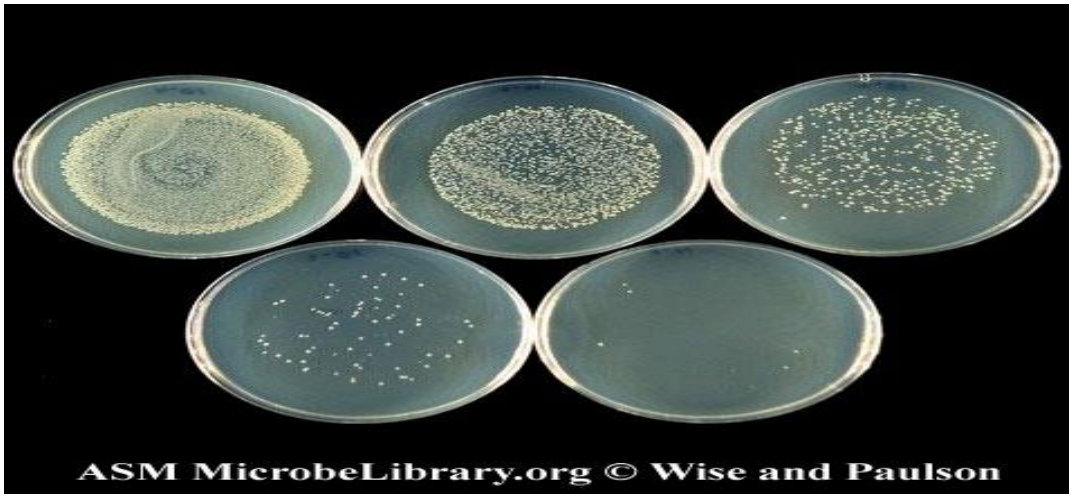


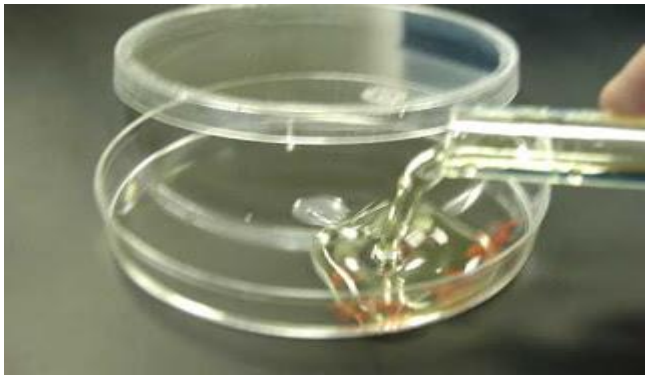
FIG. 20. Picture of spread plates showing bacterial growth (*E. coli*, 40 hours, room temperature) on five plates prepared from a ten-fold dilution series. Care was taken to avoid spreading to the edges of the plates as it is more difficult to count colonies along the edge of the agar.

Duplicate or triplicate plates with 30 to 300 CFUs/plate are used to calculate CFUs/ml.

3. Pour plate method

Pour plate Technique

A pour plate is a method of melted agar inoculation followed by petri dish incubation. A known volume (usually 0.1-1.0 ml) of culture is pipette into a sterile petri plate; melted agar medium is then added and mixed well by gently swirling the plate on the table top. Because the sample is mixed with the molten agar medium, a larger volume can be used than with the spread plate. However, with the pour plate method the organism to be counted must be able to briefly withstand the temperature of melted agar, 45^{o}C.



The cultures are inoculated into melted agar that has been cooled to 45ºC. The liquid medium is well mixed then poured into a petri dish (or vice versa*)* Colonies form within the agar matrix rather than on top as they do when streaking a plate. Pour plates are useful for quantifying microorganisms that grow in solid medium. Because the "pour plate" embeds colonies in agar it can supply a sufficiently oxygen deficient environment that it can allow the growth and quantification of microaerophiles.

4. Streak Plate Technique

For organisms that grow well on agar plate, streak plate is the method of choice for obtaining pure culture.

The key principles of this method is that, by streaking, a dilution gradient (number of cells decrease as they move across the agar and away from the point of inoculation) is established across the face of the plate as bacterial cells are deposited on the agar surface. Because of this dilution gradient, confluent growth occurs on part of the plate where the bacterial cells are not sufficiently separated; in other regions of the plate where few bacteria are deposited separate macroscopic colonies develop that can easily be seen with naked eye. Each well isolated colony is assumed to arise from a single bacterium and therefore to represent a clone of a pure culture.

Purpose of Streak Plate Technique: The purpose of the streak plate is to obtain isolated colonies from an inoculum by creating areas of increasing dilution on a single plate. Isolated colonies represent a clone of cells, being derived from a single precursor cell.

Many different streaking patterns can be used to separate individual bacterial cells on the agar surface.

Using selective media - Mannitol salt agar for staphylococcus aureus

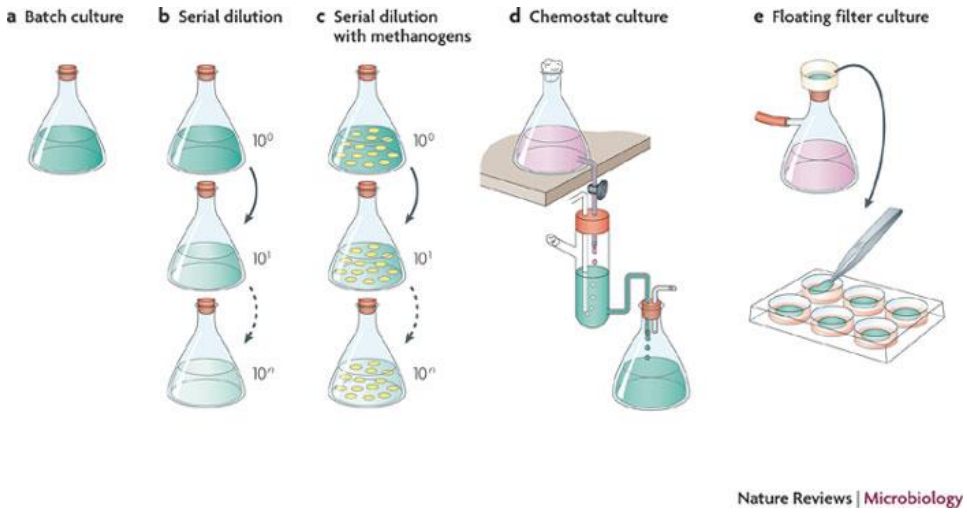
LAWN CULTURE

- Provides a uniform surface growth of the bacterium.
- Uses
  - For bacteriophage typing.
  - Antibiotic sensitivity testing.
  - In the preparation of bacterial antigens and vaccines.
- Lawn cultures are prepared by flooding the surface of the plate with a liquid suspension of the bacterium.

Batch and continuous culture

1. **batch culture** A technique used to grow microorganisms or cells. A limited supply of nutrients for growth is provided; when these are used up, or some other factor becomes limiting, the culture declines. Cells, or products that the organisms have made, can then be harvested from the culture.

In batch cultivation, the bacteria are inoculated into the bioreactor (always stirred tank bioreactor). Then, under certain conditions (temperature, pH, aeration, etc.) the bacteria go through all the growth phases (lag, exponential, stationary). At last, the fermentation is stopped and the product is collected. Then, after cleaning and sterilization of the fermenter, the fermenter is ready for another batch.



2. **In continuous cultivation**, the fresh medium flows into the fermentor continuously, and part of the medium in the reactor is withdrawn from the fermenter at the same flow rate of the inlet flow. The table below shows the advantages and disadvantages of different modes of operation of the stirred tank rector. (Ref 1.)

Mode of operation	Advantages	Disadvantages

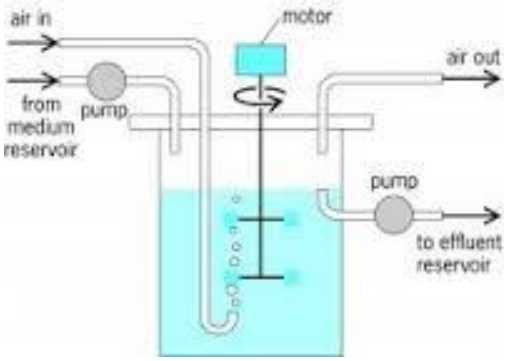
Batch	Versatile: can be used for different reactions every day. Safe: can be properly sterilized. Little risk of infection or strain mutation Complete conversion of substrate is possible	High labor cost: skilled labor is required Much idle time: Sterilization, growth of inoculum, cleaning after the fermentation Safety problems: when filling, emptying, cleaning
Continuous	Works all the time: low labor cost, good utilization of reactor Often efficient: due to the autocatalytic nature of microbial reactions, the productivity can be high. Automation may be very appealing Constant product quality	Often disappointing: promised continuous production for months fails due to a. infection. b. spontaneous mutation of microorganisms to non producing strain Inflexible: can rarely be used for other productions without substantial retrofitting

From the above comparison, although continuous culture has some disadvantage, it can outperform batch culture by eliminating the inherent down time for cleaning and sterilization and the long lags before the organisms enter a brief period of high productivity.

Continuous culture is superior to batch culture in several ways for research. Interpretation of results is difficult for batch culture because of changing concentrations of products and reactants, varying pH and redox potential, and a complicated mix of growing, dying, and dead cells. Data from continuous cultures have much less complexity because there are dynamic equilibria or small excursions from steady state. Cause and effect relationships tend to be obvious.

a. Chemostat

A **chemostat** (from chemical environment is static) is a bioreactor to which fresh medium is continuously added, while culture liquid containing left over nutrients, metabolic end products and microorganisms are continuously removed at the same rate to keep the culture volume constant.



b. Turbidostat

A **turbidostat** is a continuous microbiological culture device, similar to a chemostat or an auxostat, which has feedback between the turbidity of the culture vessel and the dilution rate.[1][2] The theoretical relationship between growth in a chemostat and growth in a turbidostat is somewhat complex, in part because they are similar. A chemostat has a fixed volume and flow rate, and thus a fixed dilution rate. A turbidostat dynamically adjusts the flow rate (and therefore the dilution rate) to make the turbidity constant. At equilibrium, operation of both the chemostat and turbidostat are identical. It is only when classical chemostat assumptions are violated (for instance, out of equilibrium; or the cells are mutating) that a turbidostat is functionally different. One case may be while cells are growing at their maximum growth rate, in which case it is difficult to set a chemostat to the appropriate constant dilution rate.[3]

While most turbidostats use a **spectrophotometer/turbidometer** to measure the optical density for control purposes, there exist other methods, such as dielectric permittivity.

3. **Fed-batch culture** is, in the broadest sense, defined as an operational technique in biotechnological processes where one or more nutrients (substrates) are fed (supplied) to the bioreactor during cultivation and in which the product(s) remain in the bioreactor until the end of the run.[1] An alternative description of the method is that of a culture in which "a base medium supports initial cell culture and a feed medium is added to prevent nutrient depletion".[2] It is also a type of **semi-batch culture**. In some cases, all the nutrients are fed into the bioreactor. The advantage of the fed-batch culture is that one can control concentration of fed-substrate in the culture liquid at arbitrarily desired levels (in many cases, at low levels).

Generally speaking, fed-batch culture is superior to conventional batch culture when controlling concentrations of a nutrient (or nutrients) affect the yield or productivity of the desired metabolite.

4. A **synchronous** or **synchronized culture** is a microbiological culture or a cell culture that contains cells that are all in the same growth stage.

Since numerous factors influence the cell cycle, some of them stochastic (random), normal, non-synchronous cultures have cells in all stages of the cell cycle. Obtaining a culture with a unified cell-cycle stage is very useful for biological research. Since cells are too small for certain research techniques, a synchronous culture can be treated as a single cell; the number of cells in the culture can be easily estimated, and quantitative experimental results can simply be divided in the number of cells to obtain values that apply to a single cell. Synchronous cultures have been extensively used to address questions regarding cell cycle and growth, and the effects of various factors on these.

Synchronous cultures can be obtained in several ways:

1. External conditions can be changed, so as to arrest growth of all cells in the culture, and then changed again to resume growth. The newly growing cells are now all starting to grow at the same stage, and they are synchronized. For example, for photosynthetic cells light can be eliminated for several hours and then re-introduced. Another method is to eliminate an essential nutrient from the growth medium and later to re-introduce it.
2. Cell growth can also be arrested using chemical growth inhibitors. After growth has completely stopped for all cells, the inhibitor can be easily removed from the culture and the cells then begin to grow synchronously. Nocodazole, for example, is often used in biological research for this purpose.
3. Cells in different growth stages have different physical properties. Cells in a culture can thus be physically separated based on their density or size, for instance. This can be achieved using centrifugation (for density) or filtration (for size).
4. In the Helmstetter-Cummings technique, a bacterial culture is filtered through a membrane. Most bacteria pass through, but some remain bound to the membrane. Fresh medium is then applied to the membrane and the bound bacteria start to grow. Newborn bacteria that detach from the membrane are now all at the same stage of growth; they are collected in a flask that now harbors a synchronous culture.

ENUMERATION OF MICROORGANISMS

I. OBJECTIVES

- To learn the different techniques used to count the number of microorganisms in a sample.
- To be able to differentiate between different enumeration techniques and learn when each should be used.
- To have more practice in serial dilutions and calculations.

II. INTRODUCTION

For unicellular microorganisms, such as bacteria, the reproduction of the cell reproduces the entire organism. Therefore, microbial growth is essentially synonymous with microbial reproduction. To determine rates of microbial growth and death, it is necessary to enumerate microorganisms, that is, to determine their numbers.

It is also often essential to determine the number of microorganisms in a given sample. For example, the ability to determine the safety of many foods and drugs depends on knowing the levels of microorganisms in those products. A variety of methods has been developed for the enumeration of microbes. These methods measure cell numbers, cell mass, or cell constituents that are proportional to cell number. The four general approaches used for estimating the sizes of microbial populations are direct and indirect counts of cells and direct and indirect measurements of microbial biomass. Each method will be described in more detail below.

1. Direct Count of Cells

Cells are counted directly under the microscope or by an electronic particle counter. Two of the most common procedures used in microbiology are discussed below.

Direct Count Using a Counting Chamber

Direct microscopic counts are performed by spreading a measured volume of sample over a known area of a slide, counting representative microscopic fields, and relating the averages back to the appropriate volume-area factors. Specially constructed counting chambers, such as the Petroff-Hauser and Levy counting chambers, simplify the direct counting procedure because they are made with depressions in which a known volume overlies an area that is ruled into squares. The ability to count a defined area

and convert the numbers observed directly to volume makes the direct enumeration procedure relatively easy.

Direct counting procedures are rapid but have the disadvantage that they do not discriminate between living and dead cells. This method is used to assess the sanitation level of a food product and in performing blood cell counts in hematology. The differential white blood cell count, which is used as an indication of the nature of a microbial infection, involves direct counting of blood cells that have been stained to differentiate different types of white blood cells.

Direct Count Using Fluorescent Dyes

Fluorescent dyes are becoming more used in recent years for a variety of procedures, one of which is bacterial counts. These dyes can be employed to stain all species, a particular species of interest in an environmental sample or even a specific component of cells.

The most widely used fluorescent dye for counting the number of bacterial cells is acridine orange which stains both living and dead cells by interacting with DNA and protein components of cells. The stained cells fluoresce orange when excited near ultraviolet light. This stain is particularly useful for determining the total number of microorganisms in samples, such as soil and water, where the co-existence of metabolically diverse populations precludes establishing conditions for the simultaneous enumeration of microbial populations by viable count procedures. The procedure is widely used in marine microbiology where population levels are often low and where viable plate counts are known to severely underestimate total number of bacteria. Typically, the viable count is less than 1% of the direct count for marine samples.

In this procedure the bacteria in a known volume of sample are stained with acridine orange and the sample is then filtered through a 0.22 μm filter. The bacteria are trapped on the filter that is then examined under a fluorescence microscope. The bacteria in a defined area of the filter are counted and the concentration in the original sample is then calculated.

Other fluorescent dyes that are also gaining popularity are cyanoditolyl tetrazolium chloride (CTC), auramine and rhodamine. CTC binds to respiration proteins in the cell and thus can demonstrate live cells. Auramine and rhodamine bind to cell wall of Mycobacteria and emit bright yellow or orange color under a fluorescent microscope. These latter stains are gradually replacing the acid-fast stain.

2. Indirect Count of Cells

Microorganisms in a sample are diluted or concentrated and grown on a suitable medium; the development of growing microorganisms (for example, colony formation on agar plates) is then used to estimate the numbers of microorganisms in the original sample.

Viable Count

The most common procedure for the enumeration of bacteria is the viable plate count. In this method, serial dilutions of a sample containing viable microorganisms are plated onto a suitable growth medium. The suspension is either spread onto the surface of agar plates (spread plate method), or is mixed with molten agar, poured into plates, and allowed to solidify (pour plate method). The plates are then incubated under conditions that permit microbial reproduction so that colonies develop that can be seen without the aid of a microscope. It is assumed that each bacterial colony arises from an individual cell that has undergone cell division. Therefore, by counting the number of colonies and accounting for the dilution factor, the number of bacteria in the original sample can be determined.

There are several drawbacks to the viable count method. The major disadvantage is that it is selective and therefore biased. The nature of the growth conditions, including the composition and pH of the medium used as well as the conditions such as temperature, determines which bacteria in a mixed population can grow. Since there is no universal set of conditions that permits the growth of all microorganisms, it is impossible to enumerate all microorganisms by viable plating. This same disadvantage, however, becomes advantageous when one is interested in only a specific microbial population. For example, we can design selective procedures for the enumeration of coliforms and other physiologically defined microbial groups.

The viable count is an estimate of the number of cells. Because some organisms exist as pairs or groups and because mixing and shaking of the sample does not always separate all the cells, we actually get a count of the "colony forming units". One cell or group of cells will produce one colony, therefore when we record results for a viable count, it is customary to record the results as colony forming units per ml (cfu/ml) or per gram (cfu/g) of test material.

Because we generally have no idea of how many bacteria are in a sample, it is almost always necessary to prepare a dilution series to ensure that we obtain a dilution containing a reasonable number of bacteria to count. Dilutions in the range 10^{-1} (1/10) to 10^{-8} (1/100,000,000) are generally used, although with particular types of samples the range of dilutions can be restricted. For example, for water that is not turbid, the maximal dilution needed is 10^{-6} because we know that if there were 10^{7} or more bacteria per milliliter, the water would be turbid.

The Most Probable Number (MPN)

The most probable number procedure dates back to the earliest days of microbiology. However, it is still widely used in sanitary bacteriology to estimate numbers of coliforms in water, milk, and other foods. Coliforms are bacteria that reside in the intestine of warm-blooded mammals and are regularly excreted in the feces. They are Gram negative rods belonging to the *Enterobacteriaceae* family, ferment lactose and produce gas. Not all members of *Enterobacteriaceae* are coliforms.

The MPN procedure is a statistical method based upon the probability theory. Samples are serially diluted to the point of extinction, that is, to a point where there are no more viable microorganisms. To detect the end point, multiple serial dilutions are inoculated into a suitable growth medium, and the development of some recognizable characteristic, such as acid production or turbidity, is used to indicate growth (the presence of at least one viable microorganism in the diluted sample). The pattern of positive tests (growth) in the replicates and statistical probability tables are used to determine the concentration (most probable number) of bacteria in the original sample. Statistical MPN tables are available for replicates of 3, 5, and 10 tubes of each dilution. The more replicate tubes used, the greater the precision of the estimate of the size of the bacterial population.

In this exercise, we will use a three-tube MPN procedure to estimate the numbers of coliforms in a water sample. As the positive criterion for identifying coliforms, we will use the ability to ferment lactose with the production of acid and gas; acid production will be detected using bromcresol purple as a pH indicator (the change from purple to yellow = acid production) and gas production will be detected using inverted Durham tubes.

3. Direct Measurement of Microbial Biomass

Cell mass is determined directly by weighing whole cells; biomass can be correlated with cell numbers by reference to a standard curve. Wet weight or dry weight of bacteria may be used for estimation of cell numbers.

4. Indirect Measurement of Microbial Biomass

Microbial biomass is estimated by measuring relatively constant biochemical components of microbial cells, such as protein, ATP, lipopolysaccharides, peptidoglycan, and chlorophyll; biomass can also be indirectly estimated by measured turbidity that can then be correlated with cell numbers by reference to a standard curve.

Various procedures based on the detection of specific microbial macromolecules or metabolic products can be used to estimate numbers of microorganisms. For example, peptidoglycan can be quantified, and because this biochemical occurs exclusively in the cell wall of bacteria, the concentration of peptidoglycan can be used to estimate bacterial numbers. Such biochemical approaches for determining bacterial numbers depend on the development of analytical chemical procedures for quantifying the particular biochemical and determining what proportion of bacterial cell is composed of the specific biochemical constituent.

III. LABORATORY SUPPLIES

Viable Plate Count

Test sample, 2 ml/test tube	1/group
BHI plates, dried at 37°C	6/group
Saline, 9.9 ml/16 mm test tube	4/group
Pipettes, 1 ml	4/group

Direct Count Using a Counting Chamber

Yeast suspension	2 ml/lab
Counting chamber	1/student
Pipettes, Pasteur	1/student

Most Probable Number Method

Test sample	same as viable count
Lactose-bromcresol purple broth	9/group
Saline tubes	same as viable count
Pipettes, 5 ml	5/group

IV. PROCEDURES (Direct count is done by each student individually; however, the students at a table will form a group to perform the viable count and the MPN.)

First Session

Direct Count Using a Counting Chamber

1. Clean a counting chamber with methanol and lens paper and then place it on the microscope stage.

2. Using the 4X objective find the ruled area on one side of the chamber and note the size and arrangements of larger squares and their small square subdivisions.

3. Shake the yeast suspension to distribute the cells evenly. Take out the counting chamber without changing the focus on the 4X objective. Place a coverslip over the calibrated surface of the counting chamber.

4. Using a transfer pipette, transfer some of the yeast suspension to the groove of the counting chamber to fill the chamber by capillary action.

5. Carefully place the counting chamber back onto the microscopic stage and observe the cells under 4X. You may need to reduce the amount of light by closing the diaphragm of the condenser to be able see the cells

6. Switch to the high-dry objective (40 X) and count the number of yeast cells in at least 50 of the small squares. If cells fall on a line, include in your count those on the top and left-hand lines and exclude those on the bottom and right-hand lines. (If the yeast cells are too dense to count, dilute your sample and start again.)

7. Calculate the average number of yeast cells per small square. Then calculate the number of yeasts per ml by dividing the average number of yeasts per small square by the volume of each small square which is 0.00025 μl. If you diluted the sample you must also multiply your results by the dilution factor to determine the

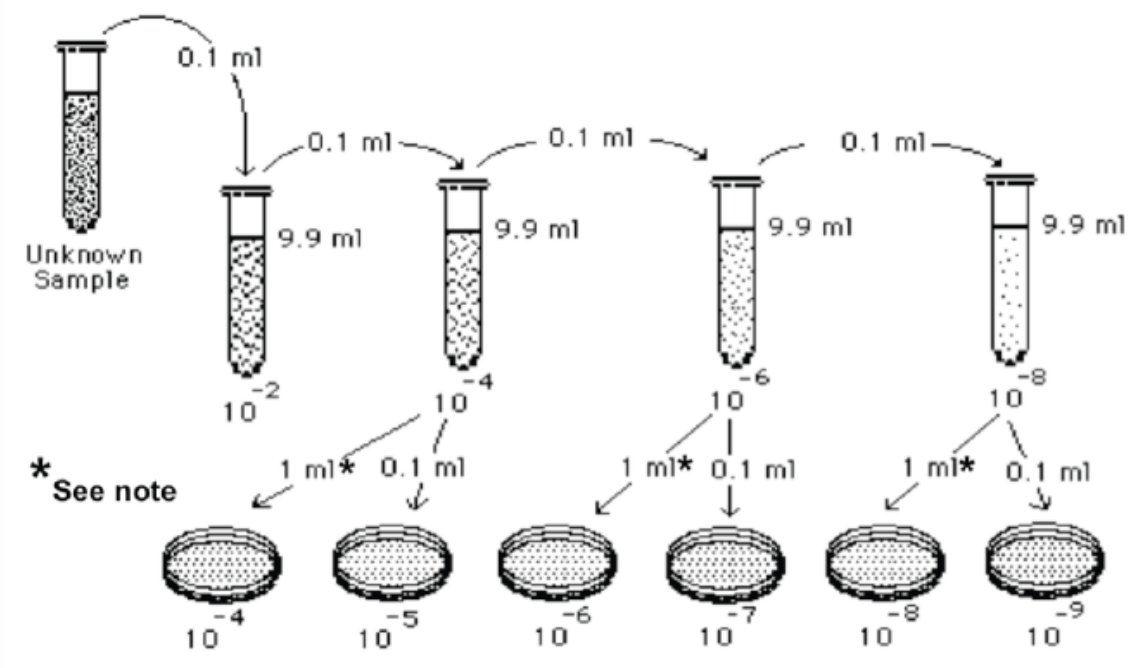
concentration of yeast cells in the original sample. Record your calculations and results.

Viable Plate Count

1. Label four 9.9 ml saline tubes 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}, respectively. Label six BHI plates 10^{-4} to 10^{-9}.

2. Vortex the unknown sample to ensure an even distribution of bacteria. Aseptically remove 0.1 ml of sample with a sterile pipette and transfer it to the 10^{-2} dilution tube (see diagram).

3. Vortex the 10^{-2} tube and transfer 0.1 ml to the 10^{-4} tube.



4. Again vortex the 10^{-4} dilution tube and transfer 0.1 ml to the 10^{-6} tube. Vortex this last tube well.

5. Vortex the 10^{-6} tube, transfer 0.1 ml to 10^{-8} tube and vortex again.

6. Using a new sterile pipette, aseptically transfer 1.0 ml from the 10^{-4} dilution tube to the plate labeled 10^{-4} and 0.1 ml to the plate labeled 10^{-5}. Spread the inoculum on the surface of the agar in each plate using an alcohol-dipped, flamed, metal spreader. Dip the spreader into the alcohol jar and quickly take it through the flame and let the alcohol burn off after each spreading. Do not allow the spreader to get too hot. Never hold the spreader in the flame for more than a second.

Aerobic and anaerobic metabolism of sugars

I. Overview of Aerobic Metabolism.

Aerobic (oxygen-using) metabolism extracts energy from carbohydrate sources, fatty acids and amino acids. While glycolysis yields two moles of ATP from one mole of glucose, full oxidation of glucose by aerobic respiration produces ~30 moles of ATP.

Aerobic metabolism occurs in three phases. First, carbohydrates are oxidized to CO_2, producing the energy-rich molecules NADH and $FADH_2$. Electrons from NADH and $FADH_2$ are then passed along the electron transport chain to the terminal electron acceptor O_2. The free energy released in electron transport is captured by coupling it to the export of protons across the mitochondrial inner membrane. Finally, the free energy of the electrochemical proton gradient is used to synthesize ATP from ADP, P_i and H^+, and to export ATP from the mitochondria.

Many catabolic pathways (carbohydrate, amino acid, fatty acid, and ketone body) converge at the TCA cycle. Many anabolic pathways depart from the TCA cycle, including synthesis of fatty acids, amino acids, purine bases, heme, cholesterol, steroid hormones and ketone bodies. The TCA cycle provides carbon skeletons in a variety of different oxidation states for these biochemical processes.

Four Coupled Reactions Constitute Aerobic Metabolism:

A. Conversion of Pyruvate to Acetyl CoA.

Pyruvate + CoA + NAD^+ ---> Acetyl CoA + CO_2 + NADH

B. The Tri-Carboxylic Acid (TCA) Cycle [Also called the Krebs Cycle and the Citric Acid Cycle].

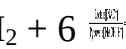
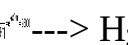
Acetyl CoA + 2 H_2O + 3 NAD^+ + FAD + GDP + P_i --->

2 CO_2 + CoA + 3 NADH + $FADH_2$ + GTP + 2 H^+

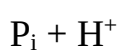
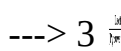
C. Electron Transport.

1/2 O_2 + H^+ + NADH + 10  ---> H_2O + NAD^+ + 10 

and

$1/2\ O_2 + FADH_2 + 6$  $---> H_2O + FAD + 6$ 

D. ATP Synthesis.

3  $+ ADP + P_i + H^+ ---> 3$  $+ ATP + H_2O$

Aerobic Metabolism in Eukaryotes Occurs in Mitochondria. Mitochondria are oval organelles ~2 mM long and ~0.5 mM in diameter. They are present in most differentiated tissues, with the exception of mature erythrocytes, cornea and lens tissue. The outer membrane is permeable to metabolites because it contains many copies of the **porin protein**. The inner membrane is impermeable to polar metabolites. Specific transporters are utilized to pass small molecules across it.

The inner membrane contains the electron transport proteins and the F_0F_1 ATP synthase. Matrix proteins include pyruvate dehydrogenase, the TCA cycle enzymes, the enzymes of fatty acid catabolism, and the enzymes that catalyze the first steps of the urea cycle. The highly folded nature of the mitochondrial inner membrane results in a high ratio of membrane surface area to matrix volume, which allows rapid exchange of NADH between the soluble enzymes of the TCA cycle and the membrane bound electron transport machinery.

Protons are pumped out of the mitochondrial matrix and into the cytosol, producing a pH gradient of 1.5 pH units and a 140 millivolt electrostatic potential difference across the inner membrane. Mitochondria act as intracellular chemical reactors, and as chemical/electrical capacitors.

Anaerobic Metabolism of Glucose

Anaerobic metabolism of glucose is a step-wise biochemical process called glycolysis or fermentation and can be performed by most cells in humans, animals and plants. Glycolysis occurs in the cytosol -- the liquid portion of the cell -- and produces energy quickly but not that efficiently. The anaerobic breakdown of 1 glucose molecule results in the production of 6 ATP molecules, 2 pyruvic acid molecules and 4 hydrogen ions, according to "Guyton and Hall Textbook of Medical Physiology." By comparison, aerobic metabolism of 1 glucose molecule results in 38 ATP molecules.

Mixed acid fermentation

The **Mixed Acid Fermentation Pathway** in *E. coli*.[1][2] End products are highlighted in blue.

Mixed acid fermentation is the biological process by which a six-carbon sugar eg. glucose is converted into a complex and variable mixture of acids. It is an anaerobic fermentation reaction that is common in bacteria. It is characteristic for members of the Enterobacteriaceae, a large family of Gram-negative bacteria that includes *E. coli*.[3]

The mixture of end products produced by mixed acid fermentation includes lactate, acetate, succinate, formate, ethanol and the gases H_2 and CO_2. The formation of these end products depends on the presence of certain key enzymes in the bacterium. The proportion in which they are formed varies between different bacterial species.[4] The mixed acid fermentation pathway differs from other fermentation pathways, which produce fewer end products in fixed amounts.

The end products of mixed acid fermentation can have many useful applications in biotechnology and industry. For instance, ethanol is widely used as a biofuel.[5] Therefore, multiple bacterial strains have been metabolically engineered in the laboratory to increase the individual yields of certain end products.[2] This research has been carried out primarily in *E. coli* and is ongoing.

Mixed Acid Fermentation in *E. coli*

E. coli use fermentation pathways as a final option for energy metabolism, as they produce very little energy in comparison to respiration.[6] Mixed acid fermentation in *E. coli* occurs in two stages. These stages are outlined by the biological database for *E. coli*, EcoCyc.[1]

The first of these two stages is a glycolysis reaction. Under anaerobic conditions, a glycolysis reaction takes place where glucose is converted into pyruvate:

glucose → 2 pyruvate

There is a net production of 2 ATP and 2 NADH molecules per molecule of glucose converted. ATP is generated by substrate-level phosphorylation. NADH is formed from the reduction of NAD.

In the second stage, pyruvate produced by glycolysis is converted to one or more end products via the following reactions. In each case, both of the NADH molecules generated by glycolysis are reoxidized to NAD^+. Each alternative pathway requires a different key enzyme in *E. coli*. After the variable amounts of different end products are formed by these pathways, they are secreted from the cell.[1]

The conversion of pyruvate to lactate is catalysed by the enzyme **lactate dehydrogenase**.

Lactate Formation

Pyruvate produced by glycolysis is converted to lactate. This reaction is catalysed by the enzyme **lactate dehydrogenase (LDHA)**.[1]

pyruvate + NADH + H^+ → **lactate** + NAD^+

Acetate Formation

Pyruvate is converted into acetyl-coenzyme A (acetyl-CoA) by the enzyme pyruvate dehydrogenase. This acetyl-CoA is then converted into acetate in *E. coli*, whilst producing ATP by substrate-level phosphorylation. Acetate formation requires two enzymes: **phosphate acetyltransferase** and **acetate kinase**.[1]

The Mixed Acid Fermentation pathway is characteristic of the **Enterobacteriaceae** family that includes *E. coli*

acetyl-CoA + phosphate → acetyl-phosphate + CoA

acetyl-phosphate + ADP → **acetate** + ATP

Ethanol Formation

Ethanol is formed in *E. coli* by the reduction of acetyl coenzyme A using NADH. This two-step reaction requires the enzyme **alcohol dehydrogenase (ADHE).**[1]

acetyl-CoA + NADH + H^+ → acetaldehyde + NAD^+ + CoA

acetaldehyde + NADH + H^+ → **ethanol** + NAD^+

Formate Formation

Formate is produced by the cleavage of pyruvate. This reaction is catalysed by the enzyme **pyruvate-formate lyase (PFL)**, which plays an important role in regulating anaerobic fermentation in *E. coli*.[7]

pyruvate + CoA → acetyl-CoA + **formate**

Succinate Formation

Succinate is formed in *E. coli* in several steps.

Phosphoenolpyruvate (PEP), a glycolysis pathway intermediate, is carboxylated by the enzyme PEP carboxylase to form oxaloacetate.[8] This is followed by the conversion of oxaloacetate to malate by the enzyme malate dehydrogenase. Fumarate hydratase then catalyses the dehydration of malate to produce fumarate.[9]

phosphoenolpyruvate + HCO_3 → oxaloacetate + phosphate

oxaloacetate + NADH + H^+ → malate + NAD^+

malate → fumarate + H_20

The final reaction in the formation of succinate is the reduction of fumarate. It is catalysed by the enzyme **fumarate reductase**.

fumarate + NADH + H^+ → **succinate** + NAD^+

This reduction is an anaerobic respiration reaction in *E. coli*, as it uses electrons associated with NADH dehydrogenase and the electron transport chain. ATP is generated by using an electrochemical gradient and ATP synthase. This is the only case in the mixed acid fermentation pathway where ATP is not produced via substrate-level phosphorylation.[1][2]

Vitamin K_2, also known as menaquinone, is very important for electron transport to fumarate in *E. coli*.