ZAPOROZHYE STATE MEDICAL UNIVERSITY

THE CHAIR OF MICROBIOLOGY, VIROLOGY, IMMUNOLOGY

Microbiological (culture) studies in the laboratory diagnosis of infectious diseases

The methodical manual
on microbiology, virology and immunology
for medical the students of II - III courses
of the medical faculty

Zaporizhzhia

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The methodical manual for practical lessons on microbiology, virology, immunology for the medical students of II-III year of the study are approved by the Central Methods Board of ZSMU as a methodical manual on practical lessons for students of the medical faculty.

The independent practical work of students is an important part of the syllabus in the course of microbiology, virology and immunology. It helps students to study this fundamental subject.

The systematic independent work enables to reach the final goal in the students' education. It is also important while preparing the students for their future clinic work with patients.

These theoretical material, questions and tests help students to get ready for examination.

МІНІСТЕРСТВО ОХОРОНИ ЗДОРОВ'Я УКРАЇНИ
ЗАПОРІЗЬКИЙ ДЕРЖАВНИЙ МЕДИЧНИЙ УНІВЕРСИТЕТ

Кафедра мікробіології, вірусології та імунології

Мікробіологічний (культуральний) метод у лабораторній діагностиці інфекційних захворівань

Навчальний посібник

для іноземних студентів II-III курсів медичних факультетів, спеціальність «Лікувальна справа»

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Мікробіологічний (культуральний) метод у лабораторній діагностиці інфекційних захворівань: навчальний посібник для іноземних студентів II-III курсів медичного факультету, спеціальність «Лікувальна справа» / Єрьоміна А.К. [та ін.]. – Запоріжжя, 2016. – 84 с.

Навчальний посібник з мікробіології, вірусології та імунології для іноземних студентів II-III курсів медичного факультету спеціальністі «Лікувальна справа».

Microbiologic Examination

Direct Examination and Techniques:

Direct examination of specimens reveals gross pathology. Microscopy may identify microorganisms.

Immunofluorescence, immuno-peroxidase staining, and other immunoassays may detect specific microbial antigens.

Genetic probes identify genus- or species-specific DNA or RNA sequences.

Culture:

Isolation of infectious agents frequently requires specialized media. Nonselective (noninhibitory) media permit the growth of many microorganisms. Selective media contain inhibitory substances that permit the isolation of specific types of microorganisms.

Microbial Identification:

Colony and cellular morphology may permit preliminary identification. Growth characteristics under various conditions, utilization of carbohydrates and other substrates, enzymatic activity, immunoassays, and genetic probes are also used.

Serodiagnosis:

A high or rising titer of specific IgG antibodies or the presence of specific IgM antibodies may suggest or confirm a diagnosis.

Antimicrobial Susceptibility:

Microorganisms, particularly bacteria, are tested in vitro to determine whether they are susceptible to antimicrobial agents.

METHODS OF BACTERIA CULTIVATION AND EXAMINATION OF THEIR QUANTITY IN TESTED MATERIAL. MEDIA AND THEIR CLASSIFICATION. TYPE AND MECHANISM OF BACTERIA NUTRITION.

1. Constructive and power (fermentation, respiration, photosynthesis) metabolism of bacteria, their interrelation (interaction). Energy of transmembrane potential and ATPh (adenosintriphosphatasa). Practical value.

2. To name types of a bacteria nutrotion and to give examples:
a - on a source of Carboneum;
b - on a source of nitrogen;
c - on a source of energy and donors of electrons;

3. To describe the mechanism of nutrition:
a – passive diffusion, facilitated diffusion, active (awake) and ionic transport;
b – the role of permeases in metabolism of bacteria;
c – the role of cytoplasmic membrane in ametabolism of bacteria.

4. Main growth factors of bacteria.

5. Enzymes of bacteria, their feature:
a - classification;
b - function;
c - practical usage.

6. To describe media:
a - classification of media;
b - main demands to media.

7. Methods of bacteria scoring in researched materials.

Metabolism refers to all the biochemical reactions that occur in a cell or organism. The study of bacterial metabolism focuses on the chemical diversity of substrate oxidations and dissimilation reactions (reactions by which substrate molecules are broken down), which normally function in bacteria to generate energy.

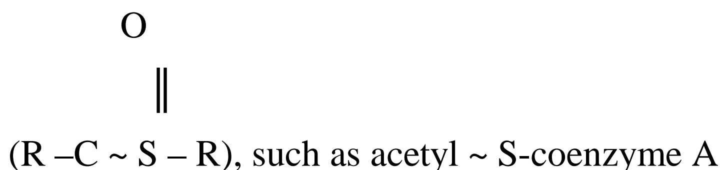
Also within the scope of the bacterial metabolism is the study of the uptake and utilization of the inorganic or organic compounds required for growth and maintenance of a cellular steady state (assimilation reactions).

These respective exergonic (energy-yielding) and endergonic (energy-requiring) reactions are catalyzed within the living bacterial cell by integrated enzyme systems, the end result being self-replication of the cell.

The capability of microbial cells to live, function, and replicate in an appropriate chemical milieu (such as a bacterial culture medium) and the chemical

changes that result during this transformation constitute the scope of bacterial metabolism.

The bacterial cell is a highly specialized energy transformer. Chemical energy generated by substrate oxidations is conserved by formation of high-energy compounds such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP) or compounds containing the thioester bond



(acetyl ~ SCoA) or succinyl ~ SCoA. ADP and ATP represent adenosine monophosphate (AMP) plus one and two high-energy phosphates (AMP ~ P and AMP ~ P~ P, respectively); the energy is stored in these compounds as high-energy phosphate bonds. In the presence of proper enzyme systems, these compounds can be used as energy sources to synthesize the new complex organic compounds needed by the cell.

All living cells must maintain steady-state biochemical reactions for the formation and use of such high-energy compounds.

From a nutritional, or metabolic, viewpoint, three major physiologic types of bacteria exist: the heterotrophs (or chemoorganotrophs), the autotrophs (or chemolithotrophs), and the photosynthetic bacteria (or phototrophs) (Table 1). These are discussed below.

Heterotrophic Metabolism

Heterotrophic bacteria, which include all pathogens, obtain energy from oxidation of organic compounds. Carbohydrates (particularly glucose), lipids, and protein are the most commonly oxidized compounds.

Biologic oxidation of these organic compounds by bacteria results in synthesis of ATP as the chemical energy source. This process also permits generation of simpler organic compounds (precursor molecules) needed by the bacteria cell for biosynthetic or assimilatory reactions.

Table 1. Nutritional Diversity Exhibited Physiologycally Different Bacteria

Required Components for Bacterial Growth				
Physiologic Type	**Carbon Sourece**	**Nitrogen Source[a] Source[b]**	**Energy Source**	**Hydrogen Source**
Heterotrophic (chemoorganotrophic)	Organic	Organic or inorganic	Oxidation of organic compounds	–
Autotrophic [a]chemolithotrophic)	CO_2	Inorganic	Oxidation of inorganic compounds	–
Photosynthetic Photolithotrophic[b] (Bacteria)	CO_2	Inorganic	Sunlight	H_2S or H_2
Cyanobacteria	CO_2	Inorganic	Sunlight	Photolysis of H_2O[c]
Photoorganotrophic (Bacteria)	CO_2	Inorganic	Sunlight	Organic compounds[d]

[a] Common inorganic nitrogen sources are NO_3^- or NH_4^+ ions; nitrogen fixers can use N_2;

[b] Many prototrophs and chemotrophs are nitrogen-fixing organisms;

[c] Results in O_2 evolution (or oxygenic photosynthesis) as commonly occurs in plants;

[d] Organic acids such as formate, acetate, and succinate can serve as hydrigen donors.

The Krebs cycle intermediate compounds serve as precursor molecules (building blocks) for the energy-requiring biosynthesis of complex organic compounds in bacteria. Degradation reactions that simultaneously produce energy and generate precursor molecules for the biosynthesis of new cellular constituents are called amphibolic.

All heterotrophic bacteria require preformed organic compounds. These carbon- and nitrogen-containing compounds are growth substrates, which are used aerobically or anaerobically to generate reducing equivalents (e.g., reduced nicotinamide adenine dinucleotide; NADH + H^+); these reducing equivalents in turn are chemical energy sources for all biologic oxidative and fermentative

systems. Heterotrophs are the most commonly studied bacteria; they grow readily in media containing carbohydrates, proteins, or other complex nutrients such as blood. Also, growth media may be enriched by the addition of other naturally occurring compounds such as milk (to study lactic acid bacteria) or hydrocarbons (to study hydrocarbon-oxidizing organisms).

Respiration

Glucose is the most common substrate used for studying heterotrophic metabolism. Most aerobic organisms oxidize glucose completely by the following reaction equation:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + \text{energy}$$

This equation expresses the cellular oxidation process called respiration. Respiration occurs within the cells of plants and animals, normally generating 38 ATP molecules (as energy) from the oxidation of 1 molecule of glucose. This yields approximately 380,000 calories (cal) per mode of glucose (ATP ~ 10,000 cal/mole).

Thermodynamically, the complete oxidation of one mole of glucose should yield approximately 688,000 cal; the energy that is not conserved biologically as chemical energy (or ATP formation) is liberated as heat (308,000 cal). Thus, the cellular respiratory process is at best about 55% efficient.

Glucose oxidation is the most commonly studied dissimilatory reaction leading to energy production or ATP synthesis. The complete oxidation of glucose may involve three fundamental biochemical pathways. The first is the glycolytic or Embden- Meyerhof-Parnas pathway, the second is the Krebs cycle (also called the citric acid cycle or tricarboxylic acid cycle), and the third is the series of membrane-bound electron transport oxidations coupled to oxidative phosphorylation.

Respiration takes place when any organic compound (usually carbohydrate) is oxidized completely to CO_2 and H_2O. In aerobic respiration, molecular O_2 serves as the terminal acceptor of electrons. For anaerobic respiration, NO_3, SO_4^{2-}, CO_2,

or fumarate can serve as terminal electron acceptors (rather than 0_2), depending on the bacterium studied. The end result of the respiratory process is the complete oxidation of the organic substrate molecule, and the end products formed are primarily CO_2 and H_2O. Ammonia is formed also if protein (or amino acid) is the substrate oxidized.

Metabolically, bacteria are unlike cyanobacteria (blue-green algae) and eukaryotes in that glucose oxidation may occur by more than one pathway. In bacteria, glycolysis represents one of several pathways by which bacteria can catabolically attack glucose. The glycolytic pathway is most commonly associated with anaerobic or fermentative metabolism in bacteria and yeasts.

In bacteria, other minor heterofermentative pathways, such as the phosphoketolase pathway, also exist.

Fermentation

Fermentation, another example of heterotrophic metabolism, requires an organic compound as a terminal electron (or hydrogen) acceptor. In fermentations, simple organic end products are formed from the anaerobic dissimilation of glucose (or some other compound). Energy (ATP) is generated through the dehydrogenation reactions that occur as glucose is broken down enzymatically.

The simple organic end products formed from this incomplete biologic oxidation process also serve as final electron and hydrogen acceptors. On reduction, these organic end products are secreted into the medium as waste metabolites (usually alcohol or acid). The organic substrate compounds are incompletely oxidized by bacteria, yet yield sufficient energy for microbial growth. Glucose is the most common hexose used to study fermentation reactions.

For most microbial fermentations, glucose dissimilation occurs through the glycolytic pathway. The simple organic compound most commonly generated is pyruvate, or a compound derived enzymatically from pyruvate, such as acetaldehyde, a-acetolactate, acetyl ~ SCoA, or lactyl ~ SCoA. Acetaldehyde can then be reduced by NADH + H+ to ethanol, which is excreted by the cell. The end product of lactic acid fermentation, which occurs in streptococci (e.g.,

Streptococcus lactis) and many lactobacilli (e.g., *Lactobacillus casei, L pentosus*), is a single organic acid, lactic acid. Organisms that ferment glucose to multiple end products, such as acetic acid, ethanol, formic acid, and CO_2, are referred to as heterofermenters. Examples of heterofermentative bacteria include *Lactobacillus, Leuconostoc,* and *Microbacterium* species. Heterofermentative fermentations are more common among bacteria, as in the mixed-acid fermentations carried out by bacteria of the family Enterobacteriaceae (e.g., *Escherichia coli, Salmonella, Shigella,* and *Proteus* species). Many of these glucose fermenters usually produce CO_2 and H_2 with different combinations of acid end products (formate, acetate, lactate, and succinate). Many obligately anaerobic clostridia (e.g., *Clostridium saccharobutyricum, C thermosaccha-rolyticum*) and *Butyribacterium* species ferment glucose with the production of butyrate, acetate, CO_2, and H_2, whereas other *Clostridum* species (*C acetobuty-licum* and *C butyricum*) also form these fermentation end products plus others (butanol, acetone, isopropanol, formate, and ethanol).

Electron Transport and Oxidative Phosphorylation

The final stage of respiration occurs through a series of oxidation-reduction electron transfer reactions that yield the energy to drive oxidative phosphorylation; this in turn produces ATP.

The enzymes involved in electron transport and oxidative phosphorylation reside on the bacterial inner (cytoplasmic) membrane. This membrane is invaginated to form structures called respiratory vesicles, lamellar vesicles, or mesosomes, which function as the bacterial equivalent of the eukaryotic mitochondrial membrane.

Respiratory electron transport chains vary greatly among bacteria, and in some organisms are absent. The respiratory electron transport chain of eukaryotic mitochondria oxidizes NADH + H+, NADPH + H+, and succinate (as well as the coacylated fatty acids such as acetyl~SCoA). The bacterial electron transport chain also oxidizes these compounds, but it can also directly oxidize, via non-pyridine

nucleotide-dependent pathways, a larger variety of reduced substrates such as lactate, malate, formate, a-glycerophosphate, H_2, and glutamate.

The respiratory electron carriers in bacterial electron transport systems are more varied than in eukaryotes, and the chain is usually branched at the site(s) reacting with molecular O_2. Some electron carriers, such as nonheme iron centers and ubiquinone (coenzyme Q), are common to both the bacterial and mammalian respiratory electron transport chains. In some bacteria, the naphthoquinones or vitamin K may be found with ubiquinone. In still other bacteria, vitamin K serves in the absence of ubiquinone. In mitochondrial respiration, only one cytochrome oxidase component is found (cytochrome *a* + *a3* oxidase).

In bacteria there are multiple cytochrome oxidases, including cytochromes *a, d, o,* and occasionally *a* + *a3* .

In bacteria cytochrome oxidases usually occur as combinations of *a1: d: o* and *a* + *a3*: *o*. Bacteria also possess mixed-function oxidases such as cytochromes P-450 and P-420 and cytochromes *c'* and *c'c'*, which also react with carbon monoxide. These diverse types of oxygen-reactive cytochromes undoubtedly have evolutionary significance. Bacteria were present before O_2 was formed; when O_2 became available as a metabolite, bacteria evolved to use it in different ways; this probably accounts for the diversity in bacterial oxygen-reactive hemoproteins.

Cytochrome oxidases in many pathogenic bacteria are studied by the bacterial oxidase reaction, which subdivides Gram-negative organisms into two major groups, oxidase positive and oxidase negative. This oxidase reaction is assayed for by using N,N,N', N'-tetramethyl-*p*-phenylenediamine oxidation (to Wurster's blue) or by using indophenol blue synthesis (with dimethyl-*p*-phenylenediamine and a-naphthol). Oxidase-positive bacteria contain integrated (cytochrome *c* type:oxidase) complexes, the oxidase component most frequently encountered is cytochrome *o*, and occasionally *a* + *a3*. The cytochrome oxidase responsible for the indophenol oxidase reaction complex was isolated from membranes of *Azotobacter vinelandii*, a bacterium with the highest respiratory rate of any known cell.

The cytochrome oxidase was found to be an integrated cytochrome *c4:o complex*, which was shown to be present in *Bacillus* species. These *Bacillus* strains are also highly oxidase positive, and most are found in morphologic group II.

Autotrophy

Bacteria that grow solely at the expense of inorganic compounds (mineral ions), without using sunlight as an energy source, are called autotrophs, chemotrophs, chemoautotrophs, or chemolithotrophs.

Like photosynthetic organisms, all autotrophs use CO_2 as a carbon source for growth; their nitrogen comes from inorganic compounds such as NH_3, NO_3^-, or N_2 (Table 4-1). Interestingly, the energy source for such organisms is the oxidation of specific inorganic compounds. Which inorganic compound is oxidized depends on the bacteria in question.

Many autotrophs will not grow on media that contain organic matter, even agar.

Also found among the autotrophic microorganisms are the sulfur-oxidizing or sulfur-compound-oxidizing bacteria, which seldom exhibit a strictly autotrophic mode of metabolism like the obligate nitrifying bacteria (see discussion of nitrogen cycle below). The representative sulfur compounds oxidized by such bacteria are H_2S, S_2, and S_2O_3. Among the sulfur bacteria are two very interesting organisms; *Thiobacillus ferrooxidans*, which gets its energy for autotrophic growth by oxidizing elemental sulfur or ferrous iron, and *T denitrificans*, which gets its energy by oxidizing S_2O_3 anaerobically, using NO_3^- as the sole terminal electron acceptor. T denitrificans reduces NO_3 to molecular N_2, which is liberated as a gas; this biologic process is called denitrification.

All autotrophic bacteria must assimilate CO_2, which is reduced to glucose from which organic cellular matter is synthesized.

The energy for this biosynthetic process is derived from the oxidation of inorganic compounds discussed in the previous paragraph.

Note that all autotrophic and phototrophic bacteria possess essentially the same organic cellular constituents found in heterotrophic bacteria; from a nutritional viewpoint, however, the autotrophic mode of metabolism is unique, occurring only in bacteria.

Anaerobic Respiration

Some bacteria exhibit a unique mode of respiration called anaerobic respiration. These heterotrophic bacteria that will not grow anaerobically unless a specific chemical component, which serves as a terminal electron acceptor, is added to the medium. Among these electron acceptors are NO_3, SO_4^2, the organic compound fumarate, and CO_2.

Bacteria requiring one of these compounds for anaerobic growth are said to be anaerobic respirers.

A large group of anaerobic respirers are the nitrate reducers. The nitrate reducers are predominantly heterotrophic bacteria that possess a complex electron transport system(s) allowing the NO3 ion to serve anaerobically as a terminal acceptor of electrons (NO_3 $\underline{2e-}$ NO_2; NO_3 $\underline{5e-}$ N_2; or NO_3 $\underline{8e-}$ NH_3). The nitrate reductase activity is common in bacteria and is routinely used in the simple nitrate reductase test to identify bacteria (see *Bergey's Manual of Deterininative Bacteriology*, 8th ed.).

The methanogens are among the most anaerobic bacteria known, being very sensitive to small concentrations of molecular O_2. They are also archaebacteria, which typically live in unusual and deleterious environments.

All of the above anaerobic respirers obtain chemical energy for growth by using these anaerobic energy-yielding oxidation reactions.

The Nitrogen Cycle

Nowhere can the total metabolic potential of bacteria and their diverse chemical-transforming capabilities be more fully appreciated than in the geochemical cycling of the element nitrogen.

All the basic chemical elements (S, O, P, C, and H) required to sustain living organisms have geochemical cycles similar to the nitrogen cycle.

The nitrogen cycle is an ideal demonstration of the ecologic interdependence of bacteria, plants, and animals.

Nitrogen is recycled when organisms use one form of nitrogen for growth and excrete another nitrogenous compound as a waste product.

This waste product is in turn utilized by another type of organism as a growth or energy substrate.

The other important biologic processes in the nitrogen cycle include nitrification (the conversion of NH_3 to NO_3 by autotrophes in the soil; denitrification (the anaerobic conversion of NO_3 to N_2 gas) carried out by many heterotrophs); and nitrogen fixation (N_2 to NH_3, and cell protein).

The latter is a very specialized prokaryotic process called diazotrophy, carried out by both free-living bacteria (such as *Azotobacter, Derxia, Beijeringeia,* and *Azomona* species) and symbionts (such as *Rhizobium* species) in conjunction with legume plants (such as soybeans, peas, clover, and bluebonnets).

All plant life relies heavily on NO3- as a nitrogen source, and most animal life relies on plant life for nutrients.

NUTRITION AND GROWTH OF BACTERIA

Every organism must find in its environment all of the substances required for energy generation and cellular biosynthesis.

The chemicals and elements of this environment that are utilized for bacterial growth are referred to as **nutrients** or **nutritional requirements**.

In the laboratory, bacteria are grown in **culture media** which are designed to provide all the essential nutrients in solution for bacterial growth.

At an elementary level, the nutritional requirements of a bacterium such as *E. coli* are revealed by the cell's elemental composition, which consists of C, H, O, N, S. P, K, Mg, Fe, Ca, Mn, and traces of Zn, Co, Cu, and Mo.

These elements are found in the form of water, inorganic ions, small molecules, and macromolecules which serve either a structural or functional role in

the cells. The general physiological functions of the elements are outlined in the Table 2.

Table 2. Major elements, their sources and functions in bacterial cells.

Element	% of dry weight	Source	Function
Carbon	50	Organic compounds or CO_2	Main constituent of cellular material.
Oxygen	20	H_2O, organic compounds, CO_2, and O_2	Constituent of cell material and cell water; O_2 is electron acceptor in aerobic respiration.
Nitrogen	14	NH_3, NO_3, organic compounds, N_2	Constituent of amino acids, nucleic acids nucleotides, and coenzymes.
Hydrogen	8	H_2O, organic compounds, H_2	Main constituent of organic compounds and cell water.
Phosphorus	3	inorganic phosphates (PO_4)	Constituent of nucleic acids, nucleotides, phospholipids, LPS, teichoic acids.
Sulfur	1	SO_4, H_2S, SO, organic sulfur compounds	Constituent of cysteine, methionine, glutathione, several coenzymes.
Potassium	1	Potassium salts	Main cellular inorganic cation and cofactor for certain enzymes.
Magnesium	0.5	Magnesium salts	Inorganic cellular cation, cofactor for certain enzymatic reactions.
Calcium	0.5	Calcium salts	Inorganic cellular cation, cofactor for certain enzymes and a component of endospores.
Iron	0.2	Iron salts	Component of cytochromes and certain nonheme iron-proteins and a cofactor for some enzymatic reactions.

The above table ignores the occurrence of trace elements in bacterial nutrition.

Trace elements are metal ions required by certain cells in such small amounts that it is difficult to detect (measure) them, and it is not necessary to add them to culture media as nutrients.

Trace elements are required in such small amounts that they are present as "contaminants" of the water or other media components. As metal ions, the trace elements usually act as cofactors for essential enzymatic reactions in the cell.

One organism's trace element may be another's required element and vice-versa, but the usual cations that qualify as trace elements in bacterial nutrition are Mn, Co, Zn, Cu, and Mo.

In order to grow in nature or in the laboratory, a bacterium must have an energy source, a source of carbon and other required nutrients, and a permissive range of physical conditions such as O_2 concentration, temperature, and pH. Sometimes bacteria are referred to as individuals or groups based on their patterns of growth under various chemical (nutritional) or physical conditions.

For example, phototrophs are organisms that use light as an energy source; anaerobes are organisms that grow without oxygen; thermophiles are organisms that grow at high temperatures.

Carbon and Energy Sources for Bacterial Growth

All living organisms require a source of energy. Organisms that use radiant energy (light) are called **phototrophs**.

Organisms that use (oxidize) an organic form of carbon are called **heterotrophs** or **chemo(hetero)trophs**.

Organisms that oxidize inorganic compounds are called **lithotrophs**.

The carbon requirements of organisms must be met by organic carbon (a chemical compound with a carbon-hydrogen bond) or by CO_2.

Organisms that use organic carbon are **heterotrophs** and organisms that use CO_2 as a sole source of carbon for growth are called **autotrophs**.

Thus, on the basis of carbon and energy sources for growth four major nutritional types of procaryotes may be defined (Table 3).

Table 3. Major nutritional types of prokaryotes

Nutritional Type	Energy Source	Carbon Source	Examples
Photoautotrophs	Light	CO_2	Cyanobacteria, some Purple and Green Bacteria
Photoheterotrophs	Light	Organic compounds	Some Purple and Green Bacteria
Chemoautotrophs or Lithotrophs (Lithoautotrophs)	Inorganic compounds, e.g. H_2, NH_3, NO_2, H_2S	CO_2	A few Bacteria and many Archaea
Chemoheterotrophs or Heterotrophs	Organic compounds	Organic compounds	Most Bacteria, some Archaea

Almost all eukaryotes are either photoautotrophic (e.g. plants and algae) or heterotrophic (e.g. animals, protozoa, fungi).

Lithotrophy is unique to procaryotes and photoheterotrophy, common in the purple and green Bacteria, occurs only in a very few eukaryotic algae.

Phototrophy has not been found in the Archaea.

This simplified scheme for use of carbon, either organic carbon or CO2, ignores the possibility that an organism, whether it is an autotroph or a heterotroph, may require small amounts of certain organic compounds for growth because they are essential substances that the organism is unable to synthesize from available nutrients.

Such compounds are called **growth factors**.

Growth factors are required in small amounts by cells because they fulfill specific roles in biosynthesis.

The need for a growth factor results from either a blocked or missing metabolic pathway in the cells.

Growth factors are organized into three categories:

1. **Purines and pyrimidines**: required for synthesis of nucleic acids (DNA and RNA);

2. **Amino acids**: required for the synthesis of proteins;

3. **Vitamins**: needed as coenzymes and functional groups of certain enzymes.

Some bacteria (e.g *E. coli*) do not require any growth factors: they can synthesize all essential purines, pyrimidines, amino acids and vitamins, starting with their carbon source, as part of their own intermediary metabolism.

Certain other bacteria (e.g. *Lactobacillus*) require purines, pyrimidines, vitamins and several amino acids in order to grow.

These compounds must be added in advance to culture media that are used to grow these bacteria.

The growth factors are not metabolized directly as sources of carbon or energy, rather they are assimilated by cells to fulfill their specific role in metabolism.

Mutant strains of bacteria that require some growth factor not needed by the wild type (parent) strain are referred to as **auxotrophs**.

Thus, a strain of *E. coli* that requires the amino acid tryptophan in order to grow would be called a tryptophan auxotroph and would be designated *E. coli* trp-.

Some vitamins that are frequently required by certain bacteria as growth factors are listed in Table 4.

The function(s) of these vitamins in essential enzymatic reactions gives a clue why, if the cell cannot make the vitamin, it must be provided exogenously in order for growth to occur.

Table 4. Common vitamins required in the nutrition of certain prokaryotes

Vitamin	Coenzyme form	Function
p-Aminobenzoic acid (PABA)		Precursor for the biosynthesis of folic acid.
Folic acid	Tetrahydrofolate	Transfer of one-carbon units and required for synthesis of thymine, purine bases, serine, methionine and pantothenate.
Biotin	Biotin	Biosynthetic reactions that require CO_2 fixation.
Lipoic acid	Lipoamide	Transfer of acyl groups in oxidation of keto acids.
Mercaptoethane-sulfonic acid	Coenzyme M	CH_4 production by methanogens.
Nicotinic acid	NAD (nicotinamide adenine dinucleotide) and NADP	Electron carrier in dehydrogenation reactions.
Pantothenic acid	Coenzyme A and the Acyl Carrier Protein (ACP)	Oxidation of keto acids and acyl group carriers in metabolism.
Pyridoxine (B6)	Pyridoxal phosphate	Transamination,deamination, decarboxylation and racemation of amino acids.
Riboflavin (B2)	FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide)	Oxidoreduction reactions.
Thiamine (B1)	Thiamine pyrophosphate (TPP)	Decarboxylation of keto acids and transaminase reactions.
Vitamin B12	Cobalamine coupled to adenine nucleoside	Transfer of methyl groups.
Vitamin K	Quinones and napthoquinones	Electron transport processes.

Culture Media for the Growth of Bacteria

For any bacterium to be propagated for any purpose it is necessary to provide the appropriate biochemical and biophysical environment.

The biochemical (nutritional) environment is made available as a **culture medium**, and depending upon the special needs of particular bacteria (as well as particular investigators) a large variety and types of culture media have been developed with different purposes and uses.

Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical and physiological properties. Nutrient media should be easily assimilable, and they should contain a known amount of nitrogen and carbohydrate substances, vitamins, a required salt concentration.

In addition they should be isotonic, and sterile, and they should have buffer properties, an optimal viscosity, and a certain oxidation-reduction potential.

The manner in which bacteria are cultivated, and the purpose of culture media, vary widely.

Liquid media are used for growth of pure batch cultures while solidified media are used widely for the isolation of pure cultures, for estimating viable bacterial populations, and a variety of other purposes.

The usual gelling agent for solid or **semisolid medium** is **agar**, a hydrocolloid derived from red algae.

Agar is used because of its unique physical properties (it melts at 100 degrees and remains liquid until cooled to 40 degrees, the temperature at which it gels) and because it cannot be metabolized by most bacteria.

Hence as a medium component it is relatively inert; it simply holds (gels) nutrients that are in aquaeous solution.

Culture media may be classified into several categories depending on their composition or use.

A **chemically-defined (synthetic) medium** is one in which the exact chemical composition is known.

Defined media are usually composed of pure biochemicals off the shelf; complex media usually contain complex materials of biological origin such as blood or milk or yeast extract or beef extract, the exact chemical composition of which is obviously undetermined.

A defined medium is a **minimal medium** if it provides only the exact nutrients (including any growth factors) needed by the organism for growth.

The use of defined minimal media requires the investigator to know the exact nutritional requirements of the organisms in question.

Chemically-defined media are of value in studying the minimal nutritional requirements of microorganisms, for enrichment cultures, and for a wide variety of physiological studies. Complex media usually provide the full range of growth factors that may be required by an organism so they may be more handily used to cultivate unknown bacteria or bacteria whose nutritional requirement are complex (i.e., organisms that require a lot of growth factors).

Most pathogenic bacteria of animals, which have adapted themselves to growth in animal tissues, require complex media for their growth.

Blood, serum and tissue extracts are frequently added to culture media for the cultivation of pathogens. Even so, for a few fastidious pathogens such as *Treponema pallidum*, the agent of syphilis, and *Mycobacterium leprae*, the cause of leprosy, artificial culture media and conditions have not been established. This fact thwarts the the ability to do basic research on these pathogens and the diseases that they cause.

Other concepts employed in the construction of culture media are the principles of selection and enrichment.

A **selective medium** is one which has a component(s) added to it which will inhibit or prevent the growth of certain types or species of bacteria and/or promote the growth of desired species.

One can also adjust the physical conditions of a culture medium, such as pH and temperature, to render it selective for organisms that are able to grow under these certain conditions.

A culture medium may also be a **differential medium** if allows the investigator to distinguish between different types of bacteria based on some observable trait in their pattern of growth on the medium.

Thus a **selective, differential medium** for the isolation of *Staphylococcus aureus*, the most common bacterial pathogen of humans, contains a very high concentration of salt (which the staph will tolerate) that inhibits most other bacteria, mannitol as a source of fermentable sugar, and a pH indicator dye.

From clinical specimens, only staphylococcus will grow. *S. aureus* is differentiated from *S. epidermidis* (a nonpathogenic component of the normal flora) on the basis of its ability to ferment mannitol. Mannitol-fermenting colonies *(S. aureus*) produce acid which reacts with the indicator dye forming a colored halo around the colonies; mannitol non-fermenters (*S. epidermidis*) use other non-fermentative substrates in the medium for growth and do not form a halo around their colonies.

An enrichment medium employs a slightly different twist. An **enrichment medium** contains some component that permits the growth of specific types or species of bacteria, usually because they alone can utilize the component from their environment.

However, an enrichment medium may have selective features. An enrichment medium for nonsymbiotic nitrogen-fixing bacteria omits a source of added nitrogen to the medium.

The medium is inoculated with a potential source of these bacteria (e.g. a soil sample) and incubated in the atmosphere wherein the only source of nitrogen available is N_2.

A selective enrichment medium for growth of the extreme halophile (*Halococcus*) contains nearly 25 percent salt [NaCl], which is required by the extreme halophile and which inhibits the growth of all other procaryotes.

Thus, nutrient media can be subdivided into three main groups:

I. Ordinary (simple) media which include meat-peptone broth, meat-peptone agar, etc.

II. Special media (serum agar, serum broth, coagulated serum, potatoes, blood agar, blood broth, etc.).

Quite often **elective media** are employed in laboratory practice in which only certain species of bacteria grow well, and other species either grow poorly or do not grow at all. Enriched media are also employed in which the species of interest to the scientist grows more intensively and more rapidly than the accompanying bacteria.

Thus, for example, on Endo's medium (elective) the growth of the Gram-positive microbes is inhibited while alkaline peptone water and alkaline meat-peptone agar serve as enriched media for the cholera vibrio. Nutrient media containing certain concentrations of penicillin are elective for penicillin-resistant strains of bacteria, but unfavourable for penicillin-sensitive strains.

III. Differential diagnostic media:

(1) media for the determination of the proteolytic action of microbes (meat-peptone gelatine);

(2) media for the determination of the fermentation of carbohydrates (Hiss media); media for the differentiation of bacteria which do and do not ferment lactose (Ploskirev, Drigalsky, Endo. etc.);

(3) media for the determination of haemolytic activity (blood agar);

(4) media for the determination of the reductive activity of microorganisms;

(5) media containing substances assimilated only by certain microbes.

Besides, in laboratory practice conservation media are used. They are used for primary seeding and transportation of the material under test.

They prevent the death of pathogenic microbes and enhance the inhibition of saprophytes.

This group of media includes a glycerin mixture composed of two parts 0.85 per cent salt solution, 1 part glycerin, and I part 15-20 per cent acid sodium phosphate, and also a glycerin preservative with lithium salts, a hypertonic salt solution, etc.

At present many nutrient media are prepared commercially as dry powders.

They are convenient to work with, are stable, and quite effective.

Non-protein media are widely used for the cultivation of bacteria, on which many heterotrophic microbes including pathogenic species grow well. The composition of these media is complex and includes a large number of components.

When cultivating in synthetic media, the use of the method of radioactive tracers has permitted a more detailed differentiation of microbes according to the character of their biosynthesis.

Selective media are widely used for differentiating prototrophic and ULixotrophic bacteria.

Prototrophs grow on a minimum medium which contains only salts and carbohydrates since they themselves are capable til' synthesizing the metaholites necessary for their development. Auxo-Irophs. in distinction, require definite media containing amino acids, vitamins, and other substances.

In consistency nutrient media may be solid (meat-peptone agar, meat-peptone gelatine, coagulated serum, potato, coagulated white of Ihc egg), semisolid (0.5 per cent meat-peptone agar), and liquid (peptone water, meat-peptone broth, sugar broth, etc.).

Physical and Environmental Requirements for Microbial Growth

The procaryotes exist in nature under an enormous range of physical conditions such as O_2 concentration, Hydrogen ion concentration (pH) and temperature.

The exclusion limits of life on the planet, with regard to environmental parameters, are always set by some microorganism, most often a procaryote, and frequently an Archaeon.

Applied to all microorganisms is a vocabulary of terms used to describe their growth (ability to grow) within a range of physical conditions.

A thermophile grows at high temperatures, an acidophile grows at low pH, an osmophile grows at high solute concentration, and so on.

This nomenclature will be employed in this section to describe the response of the procaryotes to a variety of physical conditions.

The Effect of Oxygen. Oxygen is a universal component of cells and is always provided in large amounts by H_2O. However, procaryotes display a wide range of responses to molecular oxygen O_2 (Table 6).

Table 6. Terms used to describe O_2 Relations of Microorganisms

Group	*Environment*		O_2 Effect
	Aerobic	Anaerobic	
Obligate Aerobe	Growth	No growth	Required (utilized for aerobic respiration)
Microaerophile	Growth if level not too high	No growth	Required but at levels below 0.2 atm
Obligate Anaerobe	No growth	Growth Toxic	
Facultative Anaerobe (Facultative Aerobe)	Growth	Growth	Not required for growth but utilized when available
Aerotolerant Anaerobe	Growth	Growth	Not required and not utilized

Obligate aerobes require O_2 for growth; they use O_2 as a final electron acceptor in aerobic respiration.

Obligate anaerobes (occasionally called **aerophobes**) do not need or use O_2 as a nutrient. In fact, O_2 is a toxic substance, which either kills or inhibits their growth. Obligate anaerobic procaryotes may live by fermentation, anaerobic respiration, bacterial photosynthesis, or the novel process of methanogenesis.

Facultative anaerobes (or **facultative aerobes**) are organisms that can switch between aerobic and anaerobic types of metabolism. Under anaerobic conditions (no O_2) they grow by fermentation or anaerobic respiration, but in the presence of O_2 they switch to aerobic respiration.

Aerotolerant anaerobes are bacteria with an exclusively anaerobic (fermentative) type of metabolism but they are insensitive to the presence of O_2. They live by fermentation alone whether or not O_2 is present in their environment.

The response of an organism to O_2 in its environment depends upon the occurrence and distribution of various enzymes which react with O_2 and various oxygen radicals that are invariably generated by cells in the presence of O_2.

All cells contain enzymes capable of reacting with O_2. For example, oxidations of flavoproteins by O_2 invariably result in the formation of H_20_2 (peroxide) as one major product and small quantities of an even more toxic free radical, superoxide or O_2.

Also, chlorophyll and other pigments in cells can react with O_2 in the presence of light and generate singlet oxygen, another radical form of oxygen which is a potent oxidizing agent in biological systems.

In aerobes and aerotolerant anaerobes the potential for lethal accumulation of superoxide is prevented by the enzyme superoxide dismutase (Table 7).

Table 7. Distribution of superoxide dismutase, catalase and peroxidase in procaryotes with different O_2 tolerances

Group	Superoxide dismutase	Catalase	Peroxidase
Obligate aerobes and most facultative anaerobes (e.g. Enterics)	+	+	-
Most aerotolerant anaerobes (e.g. Streptococci)	+	-	+
Obligate anaerobes (e.g. Clostridia, Methanogens, Bacteroides)	-	-	-

All organisms which can live in the presence of O_2 (whether or not they utilize it in their metabolism) contain superoxide dismutase.

Nearly all organisms contain the enzyme catalase, which decomposes H_2O_2. Even though certain aerotolerant bacteria such as the lactic acid bacteria lack catalase, they decompose H_2O_2 by means of peroxidase enzymes which derive electrons from $NADH_2$ to reduce peroxide to H_2O.

Obligate anaerobes lack superoxide dismutase and catalase and/or peroxidase, and therefore undergo lethal oxidations by various oxygen radicals when they are exposed to O_2.

All photosynthetic (and some nonphotosynthetic) organisms are protected from lethal oxidations of singlet oxygen by their possession of carotenoid pigments which physically react with the singlet oxygen radical and lower it to its nontoxic "ground" (triplet) state. Carotenoids are said to "quench" singlet oxygen radicals.

The Effect of pH on Growth. The pH, or hydrogen ion concentration, [H+], of natural environments varies from about 0.5 in the most acidic soils to about 10.5 in the most alkaline lakes (Table 8).

Table 8. Minimum, maximum and optimum pH for growth of certain prokaryotes

Organism	Minimum pH	Optimum pH	Maximum pH
Lactobacillus acidophilus	4.0-4.6	5.8-6.6	6.8
Staphylococcus aureus	4.2	7.0-7.5	9.3
Escherichia coli	4.4	6.0-7.0	9.0
Clostridium sporogenes	5.0-5.8	6.0-7.6	8.5-9.0
Erwinia caratovora	5.6	7.1	9.3
Pseudomonas aeruginosa	5.6	6.6-7.0	8.0
Streptococcus pneumoniae	6.5	7.8	8.3
Nitrobacter spp	6.6	7.6-8.6	10.0

Appreciating that pH is measured on a logarithmic scale, the [H+] of natural environments varies over a billion-fold and some microorganisms are living at the extremes, as well as every point between the extremes!

Most free-living procaryotes can grow over a range of 3 pH units, about a thousand fold change in [H+].

The range of pH over which an organism grows is defined by **three cardinal points**: the **minimum pH**, below which the organism cannot grow, the **maximum pH**, above which the organism cannot grow, and the **optimum pH**, at which the organism grows best.

For most bacteria there is an orderly increase in growth rate between the minimum and the optimum and a corresponding orderly decrease in growth rate

between the optimum and the maximum pH, reflecting the general effect of changing [H+] on the rates of enzymatic reaction.

Microorganisms which grow at an optimum pH well below neutrality (7.0) are called **acidophiles**. Those which grow best at neutral pH are called **neutrophiles** and those that grow best under alkaline conditions are called **alkaliphiles**.

Obligate acidophiles, such as some *Thiobacillus* species, actually require a low pH for growth since their membranes dissolve and the cells lyse at neutrality. Several genera of Archaea, including *Sulfolobus* and *Thermoplasma*, are obligate acidophiles. Among eukaryotes, many fungi are acidophiles, and the champion of growth at low pH is the eukaryotic alga *Cyanidium* which can grow at a pH of 0.

In the construction and use of culture media, one must always consider the optimum pH for growth of a desired organism and incorporate **buffers** in order to maintain the pH of the medium in the changing milieu of bacterial waste products that accumulate during growth.

Many pathogenic bacteria exhibit a relatively narrow range of pH over which they will grow. Most diagnostic media for the growth and identification of human pathogens have a pH near 7.

Enzymes and Their Role in Metabolism

Enzymes, organic catalysts of a highly molecular structure, are produced by the living cell. They are of a protein nature, are strictly specific in action, and play an important part in the metabolism of microorganisms.

Their specificity is associated with active centres formed by a group of amino acids.

Enzymes of microbial origin have various effects and are highly active. They have found a wide application in industry, agriculture and medicine, and are gradually replacing preparations produced by higher plants and animals.

With the help of amylase produced by mould fungi starch is saccharified and this is employed in beer making, industrial alcohol production and bread making.

Proteinases produced by microbes are used for removing the hair from hides, tanning hides, liquefying the gelatinous layer from films during regeneration, and for dry cleaning.

Fibrinolysin produced by streptococci dissolves the thrombi in human blood vessels. Enzymes which hydrolyse cellulose aid in an easier assimilation of rough fodder.

Due to the application of microbial enzymes, the medical industry has been able to obtain alkaloids, polysaccharides, and steroids (hydrocortisone, prednisone, prednisolone. etc.).

Bacteria play an important role in the treatment of caoutchouc, collon. silk. coffee, cocoa, and tobacco: significant processes lake place under their effect which change these substances essentially in the needed direction. In specific weight the synthetic capacity of microorganisms is very high.

The total weight of bacterial cytoplasm on earth is much higher than that of animal cytoplasm. The biochemical activity of microbes is of no less general biological importance than that of photosynthesis. The cessation of the existence of microorganisms would lead inevitably to the death of plants and animals.

Enzymes permit some species of microorganisms to assimilate methane. butane, and other hydrocarbons, and to synthesize complex organic compounds from them.

Thus, for example, with the help of the enzymatic ability of yeasts in special-type industrial installations protein-vitamin concentrates (PVC) can be obtained from waste products of petroleum (paraffins), which are employed in animal husbandry as a valuable nutrient substance supplementing rough fodder.

Some soil micro-organisms destroy by means of enzymes chemical substances (carcinogens) which are detrimental to the human body because they induce malignant tumours.

Some enzymes are excreted by the cell into the environment (exoenzymes) for breaking down complex colloid nutrient materials while other enzymes are contained inside the cell (endoenzymes).

Depending on the conditions of origin of enzymes there are constitutive enzymes which are constantly found in the cell irrespective of the presence of a catalysing substrate. These include the main enzymes of cellular metabolism (lipase. carbohydrase. proteinase, oxydase, etc.).

Adaptive enzymes occur only in the presence of the corresponding substrate (penicillinase, amino acid decarboxylase, alkaline phosphatase, B-galactosidase, etc.). The synthesis of induced enzymes in microbes occurs due to the presence in the cells of free amino acids and with the participation of ready proteins found in the bacteria.

According to chemical properties enzymes can be subdivided into three groups:

1 – enzymes composed only of proteins:

2 – enzymes containing in addition, to protein metallic ions essential for their activity, and assisting m the combination of the enzyme with the substrate, and taking part in the cyclic enzymatic transformations:

3 – enzymes which contain distinct organic molecules (coenzymes. prosthetic groups) essential for their activity. Some enzymes contain vitamins.

Bacterial enzymes are subdivided into some groups:

1. **Hydrolases** which catalyse the breakdown of the link between the carbon and nitrogen atoms, between the oxygen and sulphur atoms, binding one molecule of water (esterases. glucosidases, proteases. amidases, nucleases, etc.).

2. **Transferases** perform catalysis by transferring certain radicals from one molecule to another (transglucosidases, transacylases. transaminases).

3. **Oxidative enzymes** (oxyreductases) which catalyse the oxidation reduction processes (oxidases, dehydrogenases, peroxidases, catalases).

4. Isomerases and racemases play an important part in carbohydrate metabolism. They are found in most species of bacteria. Phosphohexoisomerase, galactovaldenase, phosphoglucomutase, hosphoglyceromutase pertain to the isomerases.

The absorption of food material by the cell is a rather complex process. Unicellular protozoa are characterized by a holozoic type of nutrition in which

hard food particles are swallowed, digested and converted to soluble compounds. Bacteria, algae, fungi, and plants possess a holophytik type of nutrition. They absorb nutrients in a dissolved state.

This difference, however, is not essential because the cells of protozoa, just like the cells of plant organisms, utilize nutrient substrates which are soluble in water or in the cell sap, while many bacteria and fungi can assimilate hard nutrients first splitting them by external digestion by means of exoenzymes.

During diffusion the dissolved substance is transferred from the region of higher concentration outside the cell into the bacterial cell until the concentration becomes the same. The passage of a solvent through the cytoplasmic membrane of bacteria from a region where it is less concentrated to one where it is more concentrated is performed by osmosis. The concentration gradient and osmotic power on both sides of the cytoplasmic membrane are quite different, and depend on the difference in concentration of many substances contained in the cell and nutrient medium. The transfer of dissolved substances from the nutrient medium to the cell can take place by suction together with the solvent if the membrane is sufficiently porous.

It has been established that the cellular membranes are made up of lipid and protein molecules arranged in a certain sequence. The charged groups of molecules have their ends directed towards the surface of the membrane. On these charged ends the protein layers are adsorbed, composed of protein chains forming a meshwork on the external and internal surfaces of the membrane.

The high selectivity which allows the cells to distinguish certain substances from others depends on the presence of enzymatic systems localized on the surface of bacterial cells. Due to the action of these enzymes, the insoluble substances in the membrane become soluble.

The cell membranes play an important role in metabolism. They are capable of changing rapidly their permeability to various substances and regulating in this way the entry of substances into the cell and their distribution in it, and the development of reactions in which these substances participate.

Some bacteria (Salmonella typhimurium} possess rudiments of memory. They recognize whether the medium is favourable or unfavourable to them. They 'run away' from an unfavourable one by means of flagella: when close to a favourable medium (glucose) Salmonella organisms swim to the 'bait'.

This ability to recognize the needed direction is probably accomplished by the trial-and-error method.

In the process of bacterial nutrition great importance is attached to exchange adsorption. The active transport of ions takes place due to (he difference in charges on the surface of membranes in the cell wall and the surrounding medium of the micro-organisms. Besides, the role of transporters, as has been suggested, is performed by liposoluble substances X and Y. Compounds are formed with ions of potassium and sodium (KX and NaY) which are capable of diffusing through the cell wall, while the membrane remains unpenetrable for free transporters.

Proteins concerned with the transport of amino acids have been isolated from the membranes of some microorganisms, and protein systems responsible for the transfer of certain sugars in general and glucose in particular have been revealed.

Practical Use of the Fermentative Properties of Microbes

The widespread and theoretically founded application of microbiological processes in the technology of industries involving fermentation, treatment of flax, hides, farming, and canning of many food products became possible only in the second half of the 19th century.

From the vital requirements of a vigorously developing industry, especially of the agricultural produce processing industry, there arose a need for a profound study of biochemical processes.

The investigations by Pasteur in this field were prepared to a great extent by the development of industry, organic chemistry, and other sciences.

Microorganisms take part in the cycle of nitrogen (putrefaction), carbon (fermentation), sulphur, phosphorus, iron, and other elements which are important in the vital activity of organisms.

Therapeutic muds and brine were produced as the result of the fermentative activity of definite microbial species.

Microorganisms are used as indicators for determining hydrolytic processes in seas and oceans, the soil requirements of fertilizers, and the exact amount of vitamins, amino acids and other substances which cannot be determined by chemical analytical methods.

Certain species of microorganisms synthesize antibiotics, enzymes, hormones, vitamins, and amino acids which are industrially prepared and used in medicine, veterinary practice, and agriculture.

The synthesis of proteins by means of special species of yeasts has been mastered.

Some soil bacteria are capable of rendering harmless (destroying) certain pesticides used in agriculture as well as chemical carcinogens.

Hydrogenous bacteria may be used to produce fodder protein by cultivation on urea or ammonium sulphate.

Some bacterial species are used for the control of methane in mines. Methanol, a monocarbon alcohol, is produced from methane by means of microbes.

Of great importance in medical microbiology is the utilization of the specific fermentative capacity of pathogenic bacteria for the determination of their species properties.

Many bacteria ferment carbohydrates producing acid or acid and gas, while proteins are fermented with the production of indole, ammonia, hydrogen sulphide, etc.

Fermentative properties of microbes are used in the laboratory diagnosis of infectious diseases, and in studying microbes of the soil, water.

Many bacteria ferment carbohydrates producing acid or acid and gas, while proteins are fermented with the production of indole, ammonia, hydrogen sulphide, etc.

THE MAIN METHODS AND PRINCIPLES OF ISOLATING OF PURE CULTURES.

BACTERIA REPRODUCTION. ISOLATION OF PURE CULTURE OF AEROBIC BACTERIA

Colony is bacterial cells of the same species which have grown from one bacterial cell on solid medium as isolated accumulation.

Bacteriological investigation is based on isolating a pure culture of the causal organism and its identification. The term pure culture refers to a population of microorganism of the same species isolated on a nutrient medium.

The Medium. The technique used and the type of medium selected depend upon the nature of the investigation.

In general, 3 situations may be encountered:

(1) one may need to raise a crop of cells of a particular species that is on hand;

(2) one may need to determine the numbers and types of organisms present in a given material;

(3) one may wish to isolate a particular type of microorganism from a natural source.

A. Growing Cells of a Given Species: Microorganisms observed microscopically to be growing in a natural environment may prove exceedingly difficult to grow in pure culture in an artificial medium.

Certain parasitic forms, for example, have never been cultivated outside the host.

In general, however, a suitable medium can be devised by carefully reproducing the conditions found in the organism's natural environment.

The pH, temperature, and aeration are simple to duplicate; the nutrients present the major problem.

The contribution made by the living environment is important and difficult to analyze; a parasite may require an extract of the host tissue, and a free-living form may require a substance excreted by a microorganism with which it is associated in nature.

B. Microbiologic Examination of Natural Materials: A given natural material may contain many different microenvironments, each providing a niche for a different species.

Plating a sample of the materials under one set of conditions will allow a selected group of forms to produce colonies but will cause many other types to be overlooked.

For this reason it is customary to plate out samples of the material using as many different media and conditions of incubation as is practicable, Six to 8 different culture conditions are not an unreasonable number if most of the forms present are to be discovered.

Since every type of organism present must have a chance to grow, solid media are used and crowding of colonies is avoided. Otherwise, competition will prevent some types from forming colonies.

C. Isolation of a Particular Type of Microorganism: A small sample of soil, if handled properly, will yield a different type of organism for every microenvironment present. For fertile soil (moist, aerated, rich in minerals and organic matter) this means that hundreds or even thousands of types can be isolated.

This is done by selecting for the desired type.

One gram of soil, for example, is inoculated into a flask of liquid medium that has been made up for the purpose of favoring one type of organism, eg, aerobic nitrogen fixers (Azotobacter).

In this case the medium contains no combined nitrogen and is incubated aerobically.

If cells of Azotobacter are present in the soil, they will grow well in this medium forms unable to fix nitrogen will grow only to the extent that the soil has introduced contaminating fixed nitrogen into the medium.

When the culture is fully grown, therefore, the percentage of Azotobacter in the total population will have increased greatly; the method is thus called enrichment culture.

Transfer of a sample of this culture to fresh medium will result in further enrichment of Azotobacter, after several serial transfers, the culture can be plated out on a solidified enrichment medium and colonies of Azotobacter isolated.

Liquid medium is used to permit competition and hence optimal selection, even when the desired type is represented in the soil as only a few cells in a population of millions.

Advantage can be taken of "natural enrichment." For example, in looking for kerosene oxidizers, oil-laden soil is chosen, since such soil is already an enrichment environment for such forms.

Enrichment culture, then, is a procedure whereby the medium is prepared so as to duplicate the natural environment (“niche”) of the desired microorganism, thereby selecting for it.

An important principle involved in such selection is the following: The organism selected for will be the type whose nutritional requirements are barely satisfied.

Azotobacter, for example, grows best in a medium containing organic nitrogen, but its minimum requirement is the presence of N; hence it is selected for in a medium containing N; as the sole nitrogen source.

If organic nitrogen is added to the medium, the conditions no longer select for Azotobacter but rather for a form for which organic nitrogen is the minimum requirement.

When searching for a particular type of organism in a natural material, it is advantageous to plate the organisms obtained on a differential medium if available. A differential medium is one that will cause the colonies of a particular type of organism to have a distinctive appearance.

For example, colonies of Escherichia coli have a characteristic iridescent sheen on agar containing the dyes eosin and methylene blue (EMB agar).

EMB agar containing a high concentration of one sugar will also cause organisms which ferment that sugar to form reddish colonies.

Differential media are used for such purposes as recognizing the presence of enteric bacteria in water or milk and the presence of certain pathogens in clinical specimens from patients.

Isolation of Microorganisms in Pure Culture

In order to study the properties of a given organism, it is necessary to handle it in pure culture free of all other types of organisms.

To do this, a single cell must be isolated from all other cells and cultivated in such a manner that its collective progeny also remain isolated. Several methods are available.

A. *Plating*: Unlike cells in a liquid medium, cells in or on a gelled medium are immobilized. Therefore, if few enough cells are placed in or on a gelled medium, each cell will grow into an isolated colony.

The ideal gelling agent for most microbiologic media is agar, an acidic polysaccharide extracted from certain red algae. A 1.5-2% suspension in water dissolves at 100 °C, forming a clear solution that gels at 45 °C.

Thus, a sterile agar solution can be copied to 50 °C, bacteria or other microbial cells added, and then the solution quickly cooled below 45 °C to form a gel. (Although most microbial cells are killed at 50 °C, the time-course of the killing process is sufficiently slow at this temperature to permit this procedure.)

Once gelled, agar will not again liquefy until it is heated above 80 °C, so that any temperature suitable for the incubation of a microbial culture can subsequently be used. In the pour-plate method, a suspension of cells is mixed with melted agar at 50 °C and poured into a Petri dish. When the agar solidifies, the cells are immobilized in the agar and grow into colonies.

If the cell suspension was sufficiently dilute, the colonies will be well separated, so that each has a high probability of being derived from a single cell. To make certain of this, however, it is necessary to pick a colony of the desired type, suspend it in water, and replate.

Repeating this procedure several times ensures that a pure culture will be obtained.

Alternatively, the original suspension can be streaked on an agar plate with a wire loop. As the streaking continues, fewer and fewer cells are left on the loop, and finally the loop may deposit single cells on the agar.

The plate is incubated and any well-isolated colony is then removed, resuspended in water, and again streaked on agar.

If a suspension (and not just a bit of growth from a colony or slant) is streaked, this method is just as reliable and much faster than the pour-plate method.

B. *Dilution:* A much less reliable method is that of extinction dilution. The suspension is serially diluted and samples of each dilution are plated. If only a few samples of a particular dilution exhibit growth, it is presumed that some of these cultures started from single cells.

This method is not used unless plating is for some reason impossible. An undesirable feature of this method is that it can only be used to isolate the predominant type of organism in a mixed population.

It is known that causative agents of infectious diseases in humans, animals, and the environment coexist with saprophytes and opportunistic microorganisms. Isolation of a pure culture allows the determination of morphological, cultural, biochemical, and other properties of the tested microorganism, which in turn makes it possible to identify it as a species.

The choice of the method of cultivation and the composition of the nutrient medium largely depends on the type of nutrition and respiration of microorganisms to be investigated.

This chapter deals with the main stages of isolation and identification of pure cultures of aerobic and anaerobic bacteria.

Methods of isolation and identification of individual causal organisms of infectious diseases are described in chapters devoted to their laboratory diagnosis.

Diagrammatically, the stages of isolation and identification of pure cultures of aerobic and facultative anaerobic bacteria may be presented in the following way.

Isolation and identification of a pure culture

First day

1. Microscopic examination of the tested material.
2. Streaking of the material tested onto nutrient media (solid, liquid).

Second day

1. Investigation of the cultural properties.
2. Sub-inoculation of colonies onto solid media to enrich for a pure culture.

Third day

1. Checking of the purity of the isolated culture.
2. Investigation of biochemical properties: (a) sugarlytic, (b) proteolytic.
3. Determination of antigenic properties.
4. Study of phagosensitivity, phagotyping, colicinogensitivity, colicinogenotyping, sensitivity to antibiotics, and other properties.

With regards to obtaining microorganisms in pure culture, are based on mechanical divorced of bacteria tested material inoculate onto surface media in Petri dish by bacteriological loop or pipette and after that streak plating evenly. After that again that glass spatula (don't burn through the flame) was used for streak plating onto the same second media in Petri dish.

The seeding has been done by bacteriological loop too. With that purpose in upper part of Petri dish has been made dense streaking, set free bacteriological loop from superfluous material.

After that are made paralel streaks at the last part of the agar.

Somever are applied method of laminar dilution, the matter of this method is a stiring diferrent serial dillution tested materials with melting and colling agar in tubes.

After that its are flooded into Petri dishes and put down into incubator.The tested materials are boiled of short duration or heat on 80 ^{0} C for destroy bacteria without spores.

The spores of microorganisms leave still alive and ater reinoculate this materials they are grown.

Fortner method. The agar media is divided into two parts. Onto the one part inoculate E.coli or Serratia marcescens (these microorganisms absorb

intensively oxygen) and onto second part tested material.

Closely stop up this Petri dish by parafin and put down into the thermostat. This method is used for obtainig anerobe culture.

Main Principles of the Cultivation of Microorganisms

Bacterial cultivation. In laboratory conditions microorganisms can be grown in nutrient media in incubation chambers maintained at a constant temperature. According to the type of heating, incubation chambers can be subdivided into electric, gas and kerosene.

Each incubation chamber has a thermoregulator which maintains a constant temperature. Temperature conditions are of great importance for the growth and reproduction of bacteria.

In relation to conditions of temperature all micro-organisms can be subdivided into three groups: psychrophilic (Gk. psychros cold, philein love), mesophilic (Gk. mesos intermediate), thermophilic (Gk. thermos warm).

Microorganisms may reproduce within a wide temperature regimen range of –10 to +80 °C.

Of great importance in the life activities of bacteria is the concentration of hydrogen ions in the nutrient medium, i. e. pH, which is expressed by the negative logarithm of the concentration of hydrogeons.

The pH characterizes the degree of acidity or alkalinity, from extremely acid (pH 0) to extremely alkaline (pH 14) conditions.

During evolution each microbial species adapted itself to existence within certain limits of hydrogen ion concentration beyond the range of which its life processes are unable to take place; It has been suggested that pH influences the activity of enzymes.

Depending on the pH, weak acids in an acid medium occur as molecules, and in an alkaline medium as ions.

Saprophytes can live in conditions within a wide range of a pH from 0.6 to 11.0, while pathogenic species of microbes grow within certain limits of hydrogen ion concentration.

Nutrient media should be easily assimilable, and they should contain a known amount of nitrogen and carbohydrate substances, vitamins, a required salt concentration.

In addition they should be isotonic, and sterile, and they should have buffer properties, an optimal viscosity, and a certain oxidation reduction potential.

During the whole history of microbiology nutrient media have gradually been perfected. Before Pasteur only infusions and decoctions were used as media for growing microbes.

Pasteur and Nageli introduced non-protein media for the cultivation of microbes.

Koch and Loeffler employed meat broth, peptone, and sodium chloride for growing microbes. This medium is a meat-peptone broth from which meat-peptone agar is prepared by adding 1-2 per cent industrial agar.

Agar (in Malayan - jelly) is compact fibrous material produced from some seaweed, forms in water solutions a solid gel. Agar contains 70-75% polysaccharides, 2-3% proteins and other nitrogen-containing substances, 2-4% ashes.

Main components of agar high molecular weight substances — agarose and agaropectin.

Agar dissolves in water while heating and solidifies at room temperature. It is manufactured as colourless plates or powder.

Because of the ability of agar upon cooling to give the nutrient medium a solid gel consistency, and due to its high resistance towards the microbial enzymes, it has received wide application in bacteriological techniques for preparing semisolid, solid, and dry nutrient media.

For the preparation of nutrient media M. Hottinger suggested the use of products of the tryptic breakdown of proteins which do not contain peptones, but contain the low molecular polypeptides and free amino acids.

L. Martin employed papain as an enzyme for the break-down of proteins. In recent years all the essential amino acids and vitamins used for the cultivation of bacteria have been obtained in a pure state.

Reproduction and Growth of Microorganisms

Reproduction in microbes constitutes the ability of self-multiplication, i.e. the increase in the number of individuals per unit volume.

The growth of microorganisms represents the increase of the mass of bacterial cytoplasm as a result of the synthesis of cellular material.

Bacteria reproduce by simple transverse division, vegetative reproduction, which occurs in different planes and produces many kinds of cells (clusters, chains, pairs, packets, etc.).

They also reproduce by budding, by means of the cleavage of segmented filaments, by reproducing cells similar to spores, by producing minute motile conidia.

And by conjugation, which brings us closely to the concept of sexual reproduction in bacteria DNA replication is an important condition in the process of amitotic binary fission of bacteria, the hydrogen bonds are ruptured and two DNA strands are formed, each one is contained in the daughter cells.

The single-stranded DNA are eventually linked by means of hydrogen bonds and again form double-chain DNA responsible for genetic information DNA replication and cell fission occur at a definite rate characteristic of each species.

Actinomycetes and many fungi (phycomycetes, ascomycetes, etc) reproduce predominantly by sporulation

The transverse division of bacteria is not only a process of cell division of one mother cell into two equal daughter cells, but represents d constant separation of daughter cells from the mother cell, the former in their turn become mother cells. After a certain number of generations, the mother cells age and perish.

This explanation has annulled the metaphysical concept of 'bacterial immortality'.

The rate of cell division differs among bacteria It depends on the species of microbe, the age of the culture, on the nutrient medium, temperature, concentration of carbon dioxide, and on many other factors.

The length of the generation off coli, Clostridium perfringens, Streptococcus faecalis is 15 minutes, while for the cells of a mammalian tissue culture it is 24 hours.

Thus, bacteria reproduce almost 100 times faster than cells of tissue culture The increase in the number of cells can be expressed in the following way:

0— 1— 2— 3 — 4— 5— n number of generations

The total amount of bacteria (N) after n generations will be equal to 2^n per cell of seeded material. If we take the original amount of bacteria inoculated into the nutrient medium as a single individual, and the time for one division as 30 minutes, then theoretically the total amount of bacteria produced per 24 hours would be equal $N=2^{48}$.

Upon division every 20 minutes, in 36 hours the microbial mass will be equal to 400 tons. Thermophilic microbes divide even more rapidly.

However, in natural as well as in artificial conditions, the reproduction of bacteria is of a considerably smaller scale.

It is limited by the effect of a number of environmental factors. Reproduction in bacteria conforms to certain laws.

Fig. 1 illustrates schematically the rate of reproduction of bacteria in arbitrary units, and the size of the bacterial population expressed as the logarithm of the numbers of live cells per millimeter of the medium.

There are eight principal phases of reproduction which are designated on the diagram by Roman numerals.

1. An initial stationary phase represents the time from the moment of seeding the bacteria on the nutrient medium.

Reproduction does not occur in this phase. The length of the initial stationary phase after seeding is 1-2 hours.

2. The lag phase of reproduction during which bacterial reproduction is not intensive, while the growth rate is accelerated. The second phase may last almost two hours.

3. Phase of exponential (logarithmic) growth which is characterized by a maximal division rate and decrease in cell size. The length of this period ranges from 5 to 6 hours.

4. Phase of negative growth acceleration during which the rate of bacterial reproduction ceases to be maximal, and the number of dividing cells diminishes. This phase lasts almost two hours.

5. A maximal stationary phase when the number of newly produced bacteria is almost equal to the number of dead organisms. This phase continues for two hours.

6. Accelerated death phase during which the equilibrium between the stationary phase and the bacterial death rate is interrupted. This continues for 3 hours.

7. Logarithmic death phase when the cells die at a constant rate. This continues almost 5 hours.

8. Decelerated death-rate phase in which those cells which remain alive enter a dormant state.

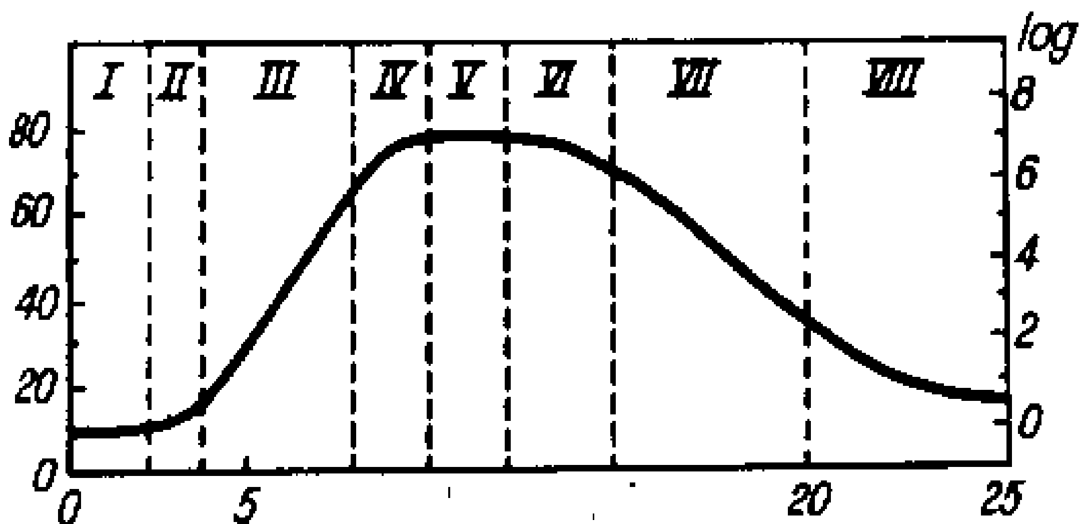


Figure 1. Graph of the reproduction of bacteria.

The length of these phases is arbitrary, as it can vary depending on the bacterial species and the conditions of cultivation.

Thus, for example, the colibacilli divide every 15-17 minutes, salmonellae of enteric fever — every 23 minutes, pathogenic streptococci — every 30 minutes, diphtheria bacilli — every 34 minutes and tubercle bacilli — every 18 hours.

Isolation and Identification of Pure Culture of Aerobic Bacteria

First day.

Prepare smears of the tested material and study them under the microscope. Then, using a spatula or a bacteriological loop, streak the material onto a solid medium in a Petri dish.

This ensures mechanical separation of microorganisms on the surface of the nutrient medium, which allows for their growth in isolated colonies.

In individual cases the material to be studied is streaked onto the liquid enrichment medium and then transferred to Petri dishes with a solid nutrient medium.

Place these dishes in a 37 ^{0}C incubator for 18-24 hrs.

Second day.

Following a 24-hour incubation, the cultural properties of bacteria (nature of their growth on solid and liquid nutrient media) are studied.

Macroscopic examination of colonies in transmitted and reflected light.

Turn the dish with its bottom to the eyes and examine the colonies in transmitted light. In the presence of various types of colonies count them and describe each of them.

The following properties are paid attention to;

(a) size of colonies (largo, 4-5 mm in diameter or more; medium, 2-4 mm; small, 1-2 mm; minute, less than 1 mm);

(b) configuration of colonies {regularly or irregularly rounded, rosette-shaped, rhizoid, etc.);

(c) degree of transparency (non-transparent, semitransparent, transparent). In a reflected light, examine the colonies from the top without opening the lid.

The following data are registered in the protocol:

(a) colour of the colonies (colourless, pigmented, the colour of the pigment);

(b) nature of the surface (smooth, glassy, moist, wrinkled, lustreless, dry, etc.);

(c) position of the colonies on the nutrient medium (protruding above the medium, submerged into the medium; flat, at the level of the medium; flattened, slightly above the medium).

Microscopic examination of colonies. Mount the dish, bottom upward, on the stage of the microscope, lower the condenser, and, using an 8 x objective, study the colonies, registering in the protocol their structure (homogeneous or amorphous, granular, fibriliar, etc.) and the nature of their edges (smooth, wavy, jagged, fringy, etc.).

Use some portion of the colonies to prepare Gram-stained smears for microscopic examination.

In the presence of uniform bacteria, transfer the remainder of colonies to an agar slant for obtaining a sufficient amount of pure culture. Place the test tubes with the inoculated medium into a 37 °C incubator for 18-24 hrs.

Third day.

Using the culture which has grown on the agar slant prepare smears and stain them by the Gram method.

Such characteristics as homogeneity of the growth, form, size, and staining of microorganisms permit definite judgement as to purity of the culture.

To identify the isolated pure culture, supplement the study of morphological, tinctorial, and cultural features with determination of their enzymatic and antigenic attributes, phago- and bacterio-cinosensitivity, toxigenicity, and other properties characterizing their species specificity.

To demonstrate carbohydrate-splitting enzymes, Hiss' media are utilized. When bacteria ferment carbohydrates with acid formation, the colour of the medium changes due to the indicator present in it.

Depending on the kind and species of bacteria studied, select media with respective mono- and disaccharides (glucose, lactose, maltose, sucrose), polysaccharides (starch, glycogen, inulin), higher alcohols (glycerol, mannitol). In the process of fermentation of the above substances aldehydes, acids, and gaseous products (CO_2, H_2, etc.) are formed.

To demonstrate proteolytic enzymes in bacteria, transfer the latter to a gelatin column. Allow the inoculated culture to stand at room temperature (20-22 °C) for several days, recording not only the development of liquefaction per se but its character as well (laminar, in the form of a nail or a fir-tree, etc.)

Proteolytic action of enzymes of microorganisms can also be observed following their streaking onto coagulated serum, with depressions forming around colonies (liquefaction).

A casein clot is split in milk to form peptone, which is manifested by the fact that milk turns yellowish (milk peptonization).

More profound splitting of protein is evidenced by the formation of indol, ammonia, hydrogen sulphide, and other compounds. To detect the gaseous substances, inoculate microorganisms into a meat-peptone broth or in a 1 per cent peptone water. Leave the inoculated cultures in an incubator for 24-72 hrs.

To demonstrate indol by Morel's method, soak narrow strips of filter paper with hot saturated solution of oxalic acid (indicator paper) and let them dry.

Place the indicator paper between the test tube wall and stopper so that it does not touch the streaked medium.

When indol is released by the 2nd-3rd day, the lower part of the paper strip turns pink as a result of its interaction with oxalic acid.

The telltale sign of the presence of ammonia is a change in the colour of a pink litmus paper fastened between the tube wall and the stopper (it turns blue). Hydrogen sulphide is detected by means of a filter paper strip saturated with lead acetate solution, which is fastened between the tube wall and the stopper.

Upon interaction between hydrogen sulphide and lead acetate the paper darkens as a result of lead sulphide formation.

To determine catalase, pour 1-2 ml of a 1 per cent hydrogen peroxide solution over the surface of a 24-hour culture of an agar slant. The appearance of gas bubbles is considered as a positive reaction. Use a culture known to contain catalase as a control.

The reduction ability of microorganisms is studied using methylene blue, thinning, litmus, indigo carmine, neutral red, etc. Add one of the above dyes to nutrient broth or agar. The medium decolorizes if the microorganism has a reduction ability.

The most widely employed is Rothberger's medium (meat-peptone agar containing 1 per cent of glucose and several drops of a saturated solution of neutral red).

If the reaction is positive, a red colour of the agar changes into yellow, yellow-green, and fluorescent, while glucose fermentation is characterized by cracks in the medium.

Antigen properties of the isolated culture are investigated by the agglutination test and other serological tests.

Species identification of aerobic bacteria is performed by comparing their morphological, cultural, biochemical, antigenic, and other properties.

ISOLATION OF PURE CULTURE OF AEROBIC BACTERIA. CULTURAL PROPETIES OF BACTERIA. TYPES OF BACTERIAL RESPIRATION. THE METHODS OF CREATION OF ANAEROBIC CONDITIONS. INOCULATION OF TESTED MATERIALS FOR ISOLATION OF PURE CULTURE OF ANAEROBIC BACTERIA

1. Cultural properties of bacteria (R - and S-forms).
2. Types and mechanism of bacterial respiration. Toxical influence of oxygen on bacteria and mechanisms of its warning.
3. Main methods of creating anaerobic conditions for cultivation of bacteria (mechanical, chemical, biological and others).
4. Media which are used for cultivation of anaerobic bacteria.

5. Stages of isolation of pure culture of anaerobic bacteria by Veinberg's and Zeissler's techniques.

On solid nutrient media microbes form colonies of different shapes and sizes which are ggregations of individuals connected by bands of cytoplasm providing for a certain structure of bacterial groupings.

The colonies may be flat. convex, dome-shaped, or pitted; their surface – smooth (S-forms). rough (R-forms). ridged, or bumpy; their edges may be straight, serralcd. fibrous, or lasseled.

The shape of the colonies also differs: e.g. round, rosette-shaped, star-shaped, tree-like.

According lo their size the colonies may be divided into large (4-5 mm in diameter), intermediate (2-4 mm), small (1-2 mm), and dwarf (less than 1 mm).

The colonies differ in their consistency, density, and colour. They may be transparent and opaque, coloured and colourless, moist, dry, and slimy.

In liquid nutrient media microbes grow producing a duffuse suspen- sion. film. or precipitate visible to the naked eye.

The growth of bacteria in the laboratory is carried out in test tubes, Petri dishes, and flasks.

In institutes for production of vaccines the cultivation of aerobes is carried out by deep stab methods. This method permits a more rational use of the nutrient substrate, and a large microbial mass can be obtained.

The cultures are grown in reactors. Aeration is produced by passing a stream of air through the medium.

The method of aeration is used in laboratory investigations to promote rapid growth of bacteria and to study some processes of metabolism.

Reproduction in microbes takes place more intensively in a flowing nutrient medium which is constantly being renewed.

For this purpose a spare tank with nutrient medium is installed, from which the mediumenters the cultivator and is carefully mixed with the culture.

Figure 2: Colonies of a different structure.

After this the excess of cultural fluid together with the suspended bacterial cells flows out. When the rate of flow of cells from the cultivator is equal to the rate of reproduction, the number of the microbial population remains constant.

Modem plant equipment is supplied with devices for automatic control over reproduction and other microbiological processes.

In usual laboratory conditions anaerobes develop in stationary or portable anaerostats containing rarefied air up to 1-8 mm or in vacuum desiccators.

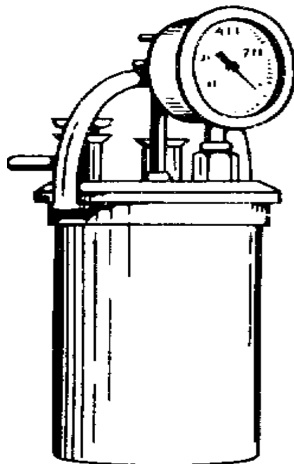


Figure 3. Portable anaerostat

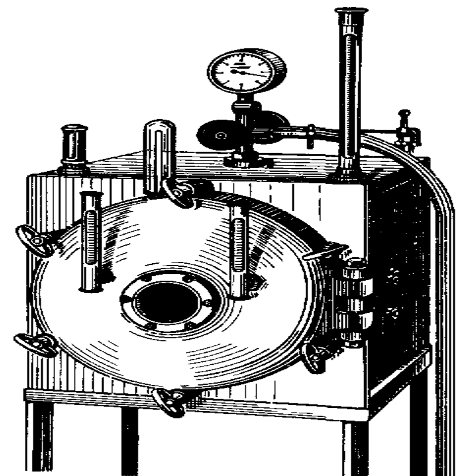


Figure 4. Stationary anaerostat

For successfully cultivating anaerobes it is necessary to seed a large amount of material into the nutrient medium. The nutrient medium should have a certain viscosity which is attained by adding 0.2 per cent agar.

The air is removed by boiling prior to seeding, and to inhibit the subsequent entry of air, the medium is covered with a layer of oil 0.5-1 cm thick.

Anaerobiosis is obtained by the adsorption of oxygen on porous substances (pumice, cotton wool, coal) and by adding reducing substances (carbohydrates, peptone, cysteine. pieces of liver, spleen, kidneys, brain, etc.).

After seeding, the test tubes are filled up with liquid vaseline. Growth of the anaerobes is usually carried out on a Kitt-Tarozzi.

Respiration in Bacteria

Respiration in bacteria is a complex process which is accompanied with the liberation of energy required by the microorganism for the synthesis of different organic compounds.

Many microbes similar to vertebrates and plants utilize the molecular oxygen in the air for respiration.

The concept of respiration as a process of oxidation of organic substances with the production of energy has undergone considerable changes due to the discovery of anaerobic microbes unable to exist in the presence of oxygen.

Pasteur established that the energy necessary for the life activity of some species of microbes is obtained in the process of fermentation (liberation of energy without the participation of oxygen).

All microbes according to type of respiration can be subdivided into obligate aerobes, facultative anaerobes and obligate anaerobes.

1. Obligate aerobes which develop well in an atmosphere containing 21 per cent of oxygen. They grow on the surfaces of liquid and solid nutrient media (brucellae, micrococci, tubercle bacilli, etc.).

2. Facultative anaerobes which can reproduce even in the absence of molecular oxygen (the majority of pathogenic and saprophytic microbes).

3. Obligate anaerobes for which the presence of molecular oxygen is a harmful growth-inhibiting factor (causative agents of tetanus, botulism, anaerobic infections, etc.).

Aerobic bacteria in the process of respiration oxidize different organic substances (carbohydrates, proteins, lipids, alcohols, organic acids, and other

compounds). During complete oxidation of one gram-molecule of glucose a definite number of calories is liberated which corresponds to the potential energy store accumulated in the carbohydrate molecule during its photosynthesis in green plants from carbon dioxide and water.

During incomplete (partial) aerobic oxidation, less energy is released corresponding to the degree of oxidation.

A typical representative of the facultative aerobes is the colibacillus which in a carbohydrate medium begins to develop first as an anaerobe breaking down the carbohydrates by fermentation.

Then it begins to utilize oxygen and grows like an aerobe, oxidizing the products of fermentation (lactic acid) farther to carbon dioxide and water. Facultative aerobes have a considerable advantage, as they can live in aerobic and anaerobic conditions.

Respiration in anaerobes takes place by fermentation of the substrate with the production of a small amount of energy.

In the fermentation of one gram-molecule of glucose considerably less energy is produced than during aerobic respiration.

The mechanism of anaerobic respiration takes place in the following way. If carbohydrates make up the oxidizing substrate, then preliminarily they are broken down with the help of auxiliary enzymes.

Thus, for example, glucose is phosphorylated employing ATP and ADP. As a result, hexose diphosphale is produced which under the influence of the enzyme aldolase breaks down into two components: phosphogly-ceraldehyde and dioxyacetone phosphate.

The latter under the effect of oxyisomerase is transformed into phosphoglyceraldehyde and later on after a sequence of reactions produces pyruvic acid.

This stage is the last in the anaerobic phase of transformation of carbon.

The later stages are specific and are completed with the production of end products.

Anaerobic processes include alcohol fermentation by yeasts, lactic acid fermentation by lactobacilli, and butyric acid fermentation by butyric acid clostridia.

Anaerobes ferment mostly nitrogen-free compounds causing fermentation. However, there is no sharp boundary between the erobic and anaerobic types of respiration.

Thus. for example, yeasts can change the anaerobic type of respiration to aerobic respiration.

First of all, they break down sugar into alcohol and carbon dioxide, and during increased aeration glucose is broken down into water and carbon dioxide.

The presence of obligate anaerobes explains the rather great adaptability of living things and the completeness of the cycle ofsubstances in nature.

It has been established by investigations that the respiration in bacteria takes place under the influence of enzymes of the oxidase and dehydrogenase types, which have a marked specificity and a multilateral activity.

The oxidase and dehydrogenase processes of respiration are closely interconnected, supplementing each other, but at the same time differing in biological role and in enzymes.

The intensity of the processes of aerobic respiration depends on the age of the culture, temperature, and nutrient substrates.

Actively growing cultures use 2500-5000 cu mm of oxygen per 1 mg of dry matter of bacleria per hour while starved cultures or cultures completely deprived of nitrogen nutrients require only 10-150 cu mm.

A young culture produces considerably more heat energy than it uses for its synthetic and other life processes. A certain part of this energy is released into the environment.

For instance, the colibacillus in the process of assimilation uses 31 per cent of the energy released, blue us bacteria – 28 per cent, Proteus vulgaris — 20 per cent, and salmonellae of enteric fever – 12 percent. The production by some microbes of

an excess of heat energy in manure, turf and garbage can ause spon- taneous heating and spontaneous combustion.

In manure and garbage dumps due to the effect of the high temperature produced by thermophilic microbes, the eggs laid by flies and also the eggs of worms are unable to develop.

Increased respiration and an increased metabolism depend on the rate of cell reproduction, on the increase of the protein synthesis in the cell, which causes an increase in the reduction properties of the medium in which the microbes develop.

Biological oxidation comprises the removal of a negatively-charged electron, reduction - the addition of a negatively-charged electron.

Between the hydrogen acceptor (yellow enzyme) and oxygen there are intermediate hydrogen carriers w hich are participants of the long chain of the catalyst of biological oxidation.

The electrons are carried by cytochromes and which are protein molecules bound with a chemical group of the haem. The haem contains an iron atom capable of undergoing oxidation and reduction alternately.

Besides cytochromes, a new substance has been discovered, a carrier of electrons, called ubiquinon or cocnzyme.

Thus the processes of respiration in bacteria are very complex and represent a long chain of a sequence of oxidation-reduction reactions with the participation of many enzyme systems transporting the electrons from the system of the most negative potential to the system of the most positive potential.

During gradual and fractional liberation of energy in respiration and during intermediate transport of hydrogen, the activity of cellular reactions increases.

The biochemical mechanisms of respiration are described in detail in biochemistry textbooks.

The habitat of microorganisms greatly influences the character of respiration. Thus, for example, upon cultivating the cholera-like vibrio in a medium containing glucose, its aerobic respiration can be decreased as a result of which it acquires the

properties of a facultative anaerobe. Yeasts are also capable of changing their type of respiration depending on the presence or absence of oxygen.

G. McLeod explained that the toxic effect of oxygen on anaerobes is due to the production of hydrogen peroxide in the presence of oxygen.

Anaerobes are unable to produce catalase. Only H,0, but not oxygen itself is toxic. However, this cannot be a complete explanation.

Anaerobes can grow if there is oxygen in the medium, which does nt kill microbes, but only inhibits their life activities. Upon the addition of reducing agents to the medium, the microbes begin to grow. as reducing agents lower the oxidation-reduction potential.

Glucose and other reducing substances act in the same way.

V. Engelhardt considers that in the presence of a high oxidation-reduction potential, the inactivation of vitally important enzymes takes place. Anaerobes then lose their ability to feed normally, and to carry out constructive processes.

Hence they perish from starvation, and not from intoxication by oxygen or H_20_2. The oxidation-reduction potential (rH,) is one of the factors on which the oxidation-reduction reactions in the nutrient medium depend.

The oxidation-reduction potential expresses the quantitative character of the degree of aerobiosis. It becomes minimal upon saturating the medium with hydrogen, and maximal upon saturating the medium with oxygen. M. dark proposed to designate the unit of the oxidation-reduction potential as rH,-the negative logarithm of the partial pressure of gaseous hydrogen.

"The range of rHs from 0 to 42,6 characterizes all degrees of saturation of an aqueous solution with hydrogen and oxygen- Aerobes exist within the limits of rH, from 14 to 20 and more, facultative aerobes from 0 to 20 and more, and anaerobes from 0 to 12.

Aerobes are adapted to existence at a higher oxidation-reduction potential, anaerobes — at a lower rH.

Anaerobes are not passive microorganisms, and they themselves cause the low rH, in the medium.

Seeded cultures of anaerobes prior to reproduction lower the rH, from 20-22 to 1-5. Thus anaerobes are characterized by a rather marked capability to adapt the medium to their requirements.

Aerobes also have these properties, and they guard themselves from an excess of oxygen by a reduction barrier.

Upon controlling the oxidation-reduction potential of the nutrient medium, conditions can be obtained for the growth of anaerobes in the presence of oxygen by lowering the rH, and also by cultivating the aerobes in anaerobic conditions by increasing the rH, of the medium.

The oxidation-reduction potential drops sharply when the bacterial culture dies, when it is lysed by a phage and when it is affected by lysozyme.

When preparing nutrient media the composition of the nutrient energy-yielding material, the reaction of the medium (pH), and its oxidation-reduction potential (rH;) are all taken into consideration.

Isolation and Identification of Pure Culture of Anaerobic Bacteria

One of the main requirements in cultivating anaerobic bacteria is removal of oxygen from the nutrient medium.

The content of oxygen can be reduced by a great variety of methods: immersing of the surface of the nutrient medium with petrolatum, introduction of microorganisms deep into a solid nutrient medium, the use of special anaerobic jars.

First day. Inoculate the studied material into Kitt-Tarozzi medium (nutrient medium): concentrated meat-peptone broth or Hottinger's broth, glucose, 0.15 per cent agar (pHl 7.2-7.4).

To adsorb oxygen, place pieces of boiled liver or minced meat to form a 1-1.5 cm layer and pieces of cotton wool on the bottom of the test tube and pour in 6-7 mi of the medium.

Prior to inoculation place the medium into boiling water for 10-20 min in order to remove air oxygen contained in it and then let it cool. Upon isolation of

spore forms of anaerobes the inoculated culture is reheated at 80 '"C for 20-30 min to kill non-sporeforming bacteria.

The cultures are immersed with petrolatum and placed into an incubator. Apart from Kitt-Tarozzi medium, liquid media containing 0.5-1 per cent glucose and pieces of animal organs, casein-acid and casein-mycotic hydrolysates can also be employed.

Casein-acid medium', casein-acid hydrolysate, 0.5 1; 10 per cent yeast extract, 0.35 1: 20 per cent corn extract, 0.15 1; millet, 240 g; cotton wool, 25 g.

The medium is poured into flasks with millet and cotton wool and sterilized for 30 min at 110 ^{0}C. Use casein-mycotic hydrolysate to obtain casein-mycotic medium.

Second day. Take note of changes in the enrichment medium, namely, the appearance of opacification or opacification in combination with gas formation. Take broth culture with a' Pasteur pipette and transfer it through a layer of petrolatum onto the bottom of the test tube.

Prepare smears on a glass slide in the usual manner, then flame fix and Gram-stain them.

During microscopic examination record the presence of Gram-positive rod forms (with or without spores).

Streak the culture from the enrichment medium onto solid nutrient media. Isolated colonies are prepared by two methods.

1. Prepare three plates with blood-sugar agar. To do it, melt and cool to 45 °C 100 ml of 2 per cent agnr on llottinger's broth, then add 10-15 ml of deftbrinated sheep or rabbit blood and 10 ml of 20 per cent sterile glucose.

Take a drop of the medium witli microorganisms into the first plate and spread it along the surface, using a glass spatula.

Use the same spatula to streak tlic culture onto tlie second and then third plates and place them into an anaerobic jar or other similar devices at 37 "C for 24-48 hrs (Zoisslcr's method).

2. Anaerobic microorganisms are grown deep in a solid nutrient medium (Veinherg's method of sequential dilutions). The culture from the medium is taken with a Pasteur pipette with a soldcd tip and transferred consecutively into the 1st, 2nd, and 3rd test tubes with 10 ml of isotonic sodium chloride solution.

Continue to dilute transferring the material into the 4th, 5th. and 6th thin-walled test tubes (0.8 cm in diameter and 18 cm in height) with melted and cooled to 50 °C meat-peptone agar or Wilson-Blair medium (to 100 ml of melted meat-peptone agar with 1 per cent glucose add 10 ml of 20 per cent sodium sulphite solution and 1 ml of 8 per cent ferric chloride).

Alter agar has solidified, place the inoculated culture into an incubator.

On the third day, study the isolated colonies formed in tlie plates and make smears from the most typical ones.

The remainder is inoculated into Kitt-Tarozzi medium. The colonies in the test tubes are removed by means of a sterile Pasteur pipette or the agar column may be pushed out of the tube by steam generated upon warming the bottom of the test tube.

Some portion of the colony is used to prepare smears, while its remainder is inoculated into Kitt-Tarozzi medium to enrich pure culture to be later identified by its morphological, cultural, biochemical, toxicogenic, antigenic, and other properties.

The Vinyale-Veyone’s method is used for mechanical protection from oxygen. The seeding are made into tube with melting and cooling (at 42 ^{0}C) agar media.

Students Practical activities:

1. To familiarize with methods of making of anaerobic conditions using anaerostat, tubes Vinyale-Vinione, Fortner biological method.

2. Macro- and microscopic examining of bacterial colonies, which grew on MPA. To describe their properties.

3. To prepare the smear from different types of colonies, stain them by Gram’s method and examine with microscope.

4. To sub-inoculate colony of E. coli onto slant agar to enrich for a pure culture.

5. To inoculate a soil into milk and Kitt-Tarozzi medium.

ENZYMES OF BACTERIA AND THEIR VALUE FOR IDENTIFICATION OF MICROORGANISMS

1. Enzymes of bacteria, their classification and practical value.

2. Identification of pure culture (morphological, tinctorial, cultural, biochemical, serological, biological).

3. What purpose has identification of pure culture?

4. Characteristic of differential diagnostic media for the determination of fermentation of the saccharolytic action of bacteria (Endo, Levin, Ploskirev, Hiss, Olkenitsky, Ressel and others).

5. How we study the peptilytic, proteolytic and hemolytic properties of bacteria?

6. What are the methods of serological and biological identification of pure culture?

Enzymes and Their Role in Metabolism

Enzymes, organic catalysts of a highly molecular structure, are produced by the living cell.

They are of a protein nature, are strictly specific in action, and play an important part in the metabolism of microorganisms.

Their specificity is associated with active centres formed by a group of amino acids.

Enzymes of microbial origin have various effect and are highly active. They have found a wide application in industry, agriculture and medicine, and are gradually replacing preparations produced by higher plants and animals.

With the help of amylase produced by mould fungi starch is saccharified and this is employed in beer making, industrial alcohol production and bread making-Proteinases produced by microbes are used for removing the hair from hides,

tanning hides, liquefying the gelatinous layer from films during regeneration, and for dry cleaning.

Fibrinolysin produced by streptococci dissolves the thrombi in human blood vessels.

Enzymes which hydrolyse cellulose aid in an easier assimilation of rough fodder.

Due to the application of microbial enzymes, the medical industry has been able to obtain alkaloids, polysaccharides, and steroids (hydrocortisone, prednisone, prednisolone. etc.).

Bacteria play an important role in the treatment of caouichouc, colon. silk. coffee, cocoa, and tobacco: significant processes lake place under their effect which change these substances essentially in the needed direction.

In specific weight the synthetic capacity of microorganisms is very high. The total weight of bacterial cytoplasm on earth is much higher than that of animal cytoplasm.

The biochemical activity of microbes is of no less general biological importance than that of photosynthesis.

The cessation of the existence of microorganisms would lead inevitably to the death of plants and animals.

Enzymes permit some species of microorganisms to assimilate methane. butane, and other hydrocarbons, and to synthesize complex organic compounds from them.

Thus, for example, with the help of the enzymatic ability of yeasts in special-type industrial installations protein-vitamin concentrates (PVC) can be obtained from waste products of petroleum (paraffin's), which are employed in animal husbandry as a valuable nutrient substance supplementing rough fodder. Some soil microorganisms destroy by means of enzymes chemical substances (carcinogens) which are detrimental to the human body because they induce malignant tumours.

Some enzymes are excreted by the cell into the environment (exoenzymes) for breaking down complex colloid nutrient materials while other enzymes are contained inside the cell (endoenzymes).

Depending on the conditions of origin of enzymes there are (onslilutire enzymes which are constantly found in the cell irrespective of the presence of a catalysing substrate. These include the main enzymes of cellular metabolism (lipase. carbohydrase. proteinase, oxydase, etc.).

Adaptive enzymes occur only in the presence of the corresponding substrate (penicillinase, amino acid decarboxylase, alkaline phosphatase.

B-galactosidase, etc.). The synthesis of induced enzymes in microbes occurs due to the presence in the cells of free amino acids and with the participation of ready proteins found in the bacteria.

According to chemical properties enzymes can be subdivided into three groups:

(1) enzymes composed only of proteins:

(2) enzymes containing in addition, to protein metallic ions essential for their activity, and assisting m the combination of the enzyme with the substrate, and taking part in the cyclic enzymatic transformations:

(3) enzymes which contain distinct organic molecules (coenzymes. prosthetic groups) essential for their activity.

Some enzymes contain vitamins.

Bacterial enzymes are subdivided into some groups:

1. Hydrolases which catalyse the breakdown of the link between the carbon and nitrogen atoms, between the oxygen and sulphur atoms, binding one molecule of water (esterases. glucosidases, proteases. amilases, nucleases, etc.).

2. Transferases perform catalysis by transferring certain radicals from one molecule to another (transglucosidases, transacylases. transaminases).

3. Oxidative enzymes (oxyreductases) which catalyse the oxidation-reduction processes (oxidases, dehydrogenases, peroxidases, catalases).

4. Isomerases and racemases play an important part in carbohydrate metabolism. They are found in most species of bacteria. Phosphohexoisomerase, galactovaldenase, phosphoglucomutase, phosphoglyceromutase pertain to the isomerases.

The absorption of food material by the cell is a rather complex process. Unicellularprotozoa are characterized by a holazoic type of nutrition in which hard food particles are swallowed, digested and converted to soluble compounds. Bacteria, algae, fungi, and plants possess a hold-photo-type of nutrition.

They absorb nutrients in a dissolved state.

This 'difference, however, is not essential because the cells of protozoa, just like the cells of plant organisms, utilize nutrient substrates which are soluble in water or in the cell sap, while many bacteria and fungi can assimilate hard nutrients first splitting them by external digestion by means of exoenzymes.

During diffusion the dissolved substance is transferred from the region of higher concentration outside the cell into the bacterial cell until the concentration becomes the same.

The passage of a solvent through the cytoplasmic membrane of bacteria from a region where it is less concentrated to one where it is more concentrated is performed by osmosis.

The concentration gradient and osmotic power on both sides of the cytoplasmic membrane are quite different, and depend on the difference in concentration of many substances contained in the cell and nutrient medium.

The transfer of dissolved substances from the nutrient medium to the cell can take place by suction together with the solvent if the membrane is sufficiently porous. It has been established that the cellular membranes are made up of lipid and protein molecules arranged in a certain sequence.

The charged groups of molecules have their ends directed towards the surface of the membrane. On these charged ends the protein layers are adsorbed, composed of protein chains forming a meshwork on the external and internal surfaces of the membrane.

The high selectivity which allows the cells to distinguish certain substances from others depends on the presence of enzymatic systems localized on the surface of bacterial cells.

Due to the action of these enzymes, the insoluble substances in the membrane become soluble.

The cell membranes play an important role in metabolism. They are capable of changing rapidly their permeability to various substances and regulating in this way the entry of substances into the cell and their distribution in it, and the development of reactions in which these substances participate.

Some bacteria (Salmonella typhimurium} possess rudiments of memory. They recognize whether the medium is favourable or unfavourable to them.

They 'run away' from an unfavourable one by means of flagella: when close to a favourable medium (glucose) Salmonella organisms swim to the 'bait'.

This ability to recognize the needed direction is probably accomplished by the trial-and-error method.

In the process of bacterial n nutrition great importance is attached to exchange adsorption.

The active transport of ions takes place due to (he difference in charges on the surface of membranes in the cell wall and the surrounding medium of the microorganisms.

Besides, the role of transporters, as has been suggested, is performed by liposoluble substances X and Y.

Compounds are formed with ions of potassium and sodium (KX and NaY) which are capable of diffusing through the cell wall, while the membrane remains unpenetrable for free transporters.

Proteins concerned with the transport of amino acids have been isolated from the membranes of some microorganisms, and protein systems responsible for the transfer of certain sugars in general and glucose in particular have been revealed.

Practical Use of the Fermentative Properties of Microbes

The widespread and theoretically founded application of microbiological processes in the technology of industries involving fermentation, treatment of flax, hides, farming, and canning of many food products became possible only in the second half of the 19th century.

From the vital requirements of a vigorously developing industry, especially of the agricultural produce processing industry, there arose a need for a profound study of biochemical processes.

The investigations by Pasteur in this field were prepared to a great extent by the development of industry, organic chemistry, and other sciences.

Microorganisms take part in the cycle of nitrogen (putrefaction), carbon (fermentation), sulphur, phosphorus, iron, and other elements which are important in the vital activity of organisms.

Therapeutic muds and brine were produced as the result of the fermentative activity of definite microbial species.

Microorganisms are used as indicators for determining hydrolytic processes in seas and oceans, the soil requirements of fertilizers, and the exact amount of vitamins, amino acids and other substances which cannot be determined by chemical analytical methods.

Certain species of microorganisms synthesize antibiotics, enzymes, hormones, vitamins, and amino acids which are industrially prepared and used in medicine, veterinary practice, and agriculture.

The synthesis of proteins by means of special species of yeasts has been mastered.

Some soil bacteria are capable of rendering harmless (destroying) certain pesticides used in agriculture as well as chemical carcinogens.

Hydrogenous bacteria may be used to produce fodder protein by cultivation on urea or ammonium sulphate.

Some bacterial species are used for the control of methane in mines. Methanol, a monocarbon alcohol, is produced from methane by means of

microbes. Of great importance in medical microbiology is the utilization of the specific fermentative capacity of pathogenic bacteria for the determination of their species properties.

Many bacteria ferment carbohydrates producing acid or acid and gas, while proteins are fermented with the production of indole, ammonia, hydrogen sulphide, etc.

Fermentative properties of microbes are used in the laboratory diagnosis of infectious diseases, and in studying microbes of the soil, water, and air.

To identify the isolated pure culture, supplement the study of morphological, tinctorial, and cultural features with determination of their enzymatic and antigenic attributes, phago- and bacterio-cinosensitivity, toxigenicity, and other properties characterizing their species specificity.

To demonstrate carbohydrate-splitting enzymes, Hiss' media are utilized. When bacteria ferment carbohydrates with acid formation, the colour of the medium changes due to the indicator present in it.

Depending on the kind and species of bacteria studied, select media with respective mono- and disaccharides (glucose, lactose, maltose, sucrose), polysaccharides (starch, glycogen, inulin), higher alcohols (glycerol, mannitol).

In the process of fermentation of the above substances aldehydes, acids, and gaseous products (CO_2, H_2, etc.) are formed.

To demonstrate proteolytic enzymes in bacteria, transfer the latter to a gelatin column.

Allow the inoculated culture to stand at room temperature (20-22 °C) for several days, recording not only the development of liquefaction per se but its character as well (laminar, in the form of a nail or a fir-tree, etc.)

Proteolytic action of enzymes of microorganisms can also be observed following their streaking onto coagulated serum, with depressions forming around colonies (liquefaction).

A casein clot is split in milk to form peptone, which is manifested by the fact that milk turns yellowish (milk peptonization).

More profound splitting of protein is evidenced by the formation of indol, ammonia, hydrogen sulphide, and other compounds.

To detect the gaseous substances, inoculate microorganisms into a meat-peptone broth or in a 1 per cent peptone water. Leave the inoculated cultures in an incubator for 24-72 hrs.

To demonstrate indol by Morel's method, soak narrow strips of filter paper with hot saturated solution of oxalic acid (indicator paper) and let them dry.

Place the indicator paper between the test tube wall and stopper so that it does not touch the streaked medium. When indol is released by the 2nd-3rd day, the lower part of the paper strip turns pink as a result of its interaction with oxalic acid.

The telltale sign of the presence of ammonia is a change in the colour of a pink litmus paper fastened between the tube wall and the stopper (it turns blue). Hydrogen sulphide is detected by means of a filter paper strip saturated with lead acetate solution, which is fastened between the tube wall and the stopper.

Upon interaction between hydrogen sulphide and lead acetate the paper darkens as a result of lead sulphide formation.

To determine catalase, pour 1-2 ml of a 1 per cent hydrogen peroxide solution over the surface of a 24-hour culture of an agar slant. The appearance of gas bubbles is considered as a positive reaction. Use a culture known to contain catalase as a control.

The reduction ability of microorganisms is studied using methylene blue, thionine, litmus, indigo carmine, neutral red, etc. Add one of the above dyes to nutrient broth or agar. The medium decolourizes if the microorganism has a reduction ability.

The most widely employed is Rothberger's medium (meat-peptone agar containing 1 per cent of glucose and several drops of a saturated solution of neutral red). If the reaction is positive, a red colour of the agar changes into yellow, yellow-green, and fluorescent, while glucose fermentation is characterized by cracks in the medium.

Antigen properties of the isolated culture are investigated by the agglutination test and other serological tests.

Species identification of aerobic bacteria is performed by comparing their morphological, cultural, biochemical, antigenic, and other properties. Phagosensitivity test is used as one of the means useful in determining the species and genus of the bacteria studied.

All immunological tests are based on specific antibody-antigen interaction. These tests are called serological since to make them one should use antibody-containing sera.

Serological tests are employed in the following cases: (a) to determine an unknown antigen (bacterium, virus, toxin) with the help of a known antibody; (b) to identify an unknown antibody (in blood serum) with the help of a known antigen.

Hence, one component (ingredient) in serological tests should always be a known entity.

The main serological tests include tests of agglutination, precipitation, lysis, neutralization, and their various modifications.

Biological examination. Biological study consists of infecting animals for the purpose of isolating the culture of the causative agents and their subsequent examination for pathogenicity and virulence.

Choice of experimental animals depends on the aim of the study. Most frequently used are rabbits, guinea pigs, albino mice, and albino rats.

This is explained by the fact that they are susceptible to the causative agents of various infections diseases in man, easy to handle, and propagate readily. Hamsters, polecats, cotton rats, monkeys, birds, etc. may also be occasionally infected.

Specialized, particularly virological, laboratories, make use of genetically standardized, so-called inbred animals (mice, rabbits, guinea pigs, and others).

Working with experimental animals, one should keep it in mind that they may have spontaneous bacterial and viral diseases and latent infections activated as

a result of additional artificial inoculation. This hinders the isolation of pure culture of the causative agent and determination of its aetiological role. Gnotobiotes (without microflora) and animals free of pathogenic microorganisms have no such drawback.

Currently they include chickens, rats, mice, guinea pigs, pigs, etc.

Laboratory animals are distinguished by their species, age, and individual sensitivity toward microorganisms. Thus, in selecting animals for study it is necessary to take into account their species and age.

For instance, sensitivity in mongrel animals may show considerable individual variations. The use of inbred animals with a definite constant susceptibility toward microorganisms excludes individual variations in sensitivity and allows for reproducible results.

Animals are infected for .isolating pure culture of the causative agent in cases where it is impossible to obtain it by any other method (for example, in contamination of the studied objects by extraneous microflora which inhibits growth of the causative agent and in case of insignificant amounts of microorganisms or their transformation into filtering forms).

Thus, in studying decayed corpses of rodents for the presence of plague causative agents, one inoculates (with suspension of the organs or blood) guinea pigs which die 3-7 days later with manifestations of septicaemia.

Pure culture of the causative agent is readily isolated from the blood of internal organs.

Contamination of susceptible animals for reproducing the infectious process is used in diseases caused by Rickettsia and viruses.

Injection to mice of material from a patient with tickborne encephalitis brings about paralysis and death in these animals.

To determine pathogenicity and virulence of the causative agents of plague, tularaemia, botulism, anthrax, and some viral diseases, cultures obtained from patients arc inoculated into albino mice, guinea pigs. rats, or suckling mice.

Students Practical activities:

1. Checking of the purity of the isolated culture, which has grown on slant agar, making smear, staining by Gram method. Sketching in album.

2. Sub-inoculating culture on Hiss medium, Olkenitsky medium and MPB for determination of saccharolytic and peptolytic properties of bacteria.

3. Perfoming presumptive agglutination test on glass with pure culture and specific serums.

4. Preparing smears from cultures on Kitt-Tarozzi medium and milk, staining by Gram method.

5. Sub-inoculating tested material from Kitt-Tarozzi medium into Vinyale-Vinone tubes for obtaining separate colonies.

6. To familiarize with growth character of E.coli and Salmonella onto Endo, Levin media.

METHODS OF STERILIZATION AND DISINFECTION.

THE PROCESSING METHODS OF ARMS OF MEDICAL PERSONAL.

INFLUENCE OF ENVIRONMENTAL FACTORS ON MICROORGANISMS

1. Effect of physical factors on microorganisms: temperature, drying up, radiant energy, high-pressure, ultrasonic sound, mechanical concussion. Concept of a temperature optimum, maximum and minimum for bacteria.

2. Effect of chemical factors on microorganisms.

3. Effect of biological factors on microorganisms. The forms of symbiose: commensalism, mutualism, parasitism. A metabiosis, satelism, synergy, antagonism.

4. Methods of a biological objects' decontamination. Sterilization, pasteurization, disinfection, asepsis, antiseptic.

5. Methods of sterilization. Sterilization of pair it is under pressure, fluid heat, dry heat, ionizing and UV radiation. Sterilization by filtrating ("cool sterilization"). The frying (burning) through torch flame, boiling.
6. Controls of sterilization.
7. Method of treating arms of surgical staff of hospital.

Influence of Environmental Factors on Microbes Effect of Physical Factors

The effect of temperature. Microbes can withstand low temperatures fairly well. The cholera vibrio does not lose its viability at a temperature of -32°C.

Some species of bacteria remain viable at a temperature of liquid air (190°C) and of liquid hydrogen (- 253°C).

Diphtheria bacilli are able to withstand freezing for three months and enteric fever bacteria are able to live long in ice. Bacillus spores withstand a temperature of –253°C for 3 days.

Many microorganisms remain viable at low temperatures, and viruses are especially resistant to low temperatures.

Thus, for example, the virus of Japanese encephalitis in a 10 per cent brain suspension does not lose its pathogenicity at -70°C over a period of one year, the causative agents of influenza and trachoma at -70 C for 6 months and Coxsackie virus at —WC for 1.5 years. Low temperatures halt putrefying and fermentative processes.

In sanitary-hygienic practice ice, cellars, and refrigerators for the storage of food products arc used according to this principle.

Only certain species of pathogenic bacteria are very sensitive to low temperatures (e. g. meningococcus, gonococcus, etc.).

During short periods of cooling these species perish quite rapidly. This is taken into account in laboratory diagnosis, and materials under test for the presence of meningitis or gonorrhoea are conveyed to the laboratory protected from cold.

At low temperatures the processes of metabolism are inhibited, the bacteria die off as a result of ageing and starvation, and the cells are destroyed under the

effect of the formation of ice crystals during freezing. Alternate high and low temperatures are lethal to microbes. It has been established, for instance, that sudden freezing as well as sudden healing causes a decrease in the life activities of pathogenic microbes.

Most asporogenic bacteria perish at a temperature of 58-60 ^{0}C within 30-60 minutes. Bacillus spores are more resistant than vegetative cells.

They withstand boiling from a few minutes to 3 hours, but perish under the effect of dry heat at 160-170°C in 1.0-1.5 hours.

Heating at 120.6°C at 2 aim steam pressure kills them within 20-30 minutes, Individual and specific variations in the resistance of microbes to high temperatures have different limits and a rather large temperature range.

The inhibition of the activity of catalase. oxydase, dehydrogenase, protein denaturation, and an interruption of the osmotic barrier are the principles of the bacterial action of high temperatures.

High temperatures cause a rather rapid destruction of viruses, but some of them (viruses of infectious hepatitis. poliomyelitis, etc.) are resistant to environmental factors.

They remain viable long in water, in the faeces of sick people or carriers, and are resistant to heat at 60°C and to small concentrations of chlorine in water.

The Effect of Temperature on Growth. Microorganisms have been found growing in virtually all environments where there is liquid water, regardless of its temperature.

In 1966, Professor Thomas D. Brock at Indiana University, made the amazing discovery in boiling hot springs of Yellowstone National Park that bacteria were not just surviving there, they were growing and flourishing.

Boiling temperature could not inactivate any essential enzyme. Subsequently, procaryotes have been detected growing around black smokers and hydrothermal vents in the deep sea at temperatures at least as high as 115 degrees. Microorganisms have been found growing at very low temperatures as well.

In supercooled solutions of H_2O as low as -20 degrees, certain organisms can extract water for growth, and many forms of life flourish in the icy waters of the Antarctic, as well as household refrigerators, near 0 degrees.

A particular microorganism will exhibit a range of temperature over which it can grow, defined by three cardinal points in the same manner as pH.

Considering the total span of temperature where liquid water exists, the procaryotes may be subdivided into several subclasses on the basis of one or another of their cardinal points for growth.

For example, organisms with an optimum temperature near 37 degrees (the body temperature of warm-blooded animals) are called **mesophiles** (Table 9.).

Table 9. Terms used to describe microorganisms in relation to temperature requirements for growth

Group	Minimum	Optimum	Maximum	Comments
Psychrophile	Below 0	10-15	Below 20	Grow best at relatively low T
Psychrotroph	0	15-30	Above 25	Able to grow at low T but prefer moderate T
Mesophile	10-15	30-40	Below 45	Most bacteria esp. those living in association with warm-blooded animals
Thermophile	45	50-85	Above 100 (boiling)	Among all thermophiles is wide variation in optimum and maximum T

Organisms with an optimum T between about 45 degrees and 70 degrees are **thermophiles**. Some Archaea with an optimum T of 80 degrees or higher and a maximum T as high as 115 degrees, are now referred to as **extreme thermophiles** or **hyperthermophiles**.

The cold-loving organisms are **psychrophiles** defined by their ability to grow at 0 degrees. A variant of a psychrophile (which usually has an optimum T of 10-15 degrees) is a **psychrotroph**, which grows at 0 degrees but displays an optimum

T in the mesophile range, nearer room temperature. Psychrotrophs are the scourge of food storage in refrigerators since they are invariably brought in from their mesophilic habitats and continue to grow in the refrigerated environment where they spoil the food.

Of course, they grow slower at 2 degrees than at 25 degrees. Think how fast milk spoils on the counter top versus in the refrigerator.

Psychrophilic bacteria are adapted to their cool environment by having largely unsaturated fatty acids in their plasma membranes.

Some psychrophiles, particularly those from the Antarctic have been found to contain polyunsaturated fatty acids, which generally do not occur in procaryotes.

The degree of unsaturation of a fatty acid correlates with its solidification T or thermal transition stage (i.e., the temperature at which the lipid melts or solidifies); unsaturated fatty acids remain liquid at low T but are also denatured at moderate T; saturated fatty acids, as in the membranes of thermophilic bacteria, are stable at high temperatures, but they also solidify at relatively high T.

Thus, saturated fatty acids (like butter) are solid at room temperature while unsaturated fatty acids (like canola oil) remain liquid in the refrigerator.

Whether fatty acids in a membrane are in a liquid or a solid phase affects the fluidity of the membrane, which directly affects its ability to function. Psychrophiles also have enzymes that continue to function, albeit at a reduced rate, at temperatures at or near 0 degrees.

Usually, psychrophile proteins and/or membranes, which adapt them to low temperatures, do not function at the body temperatures of warm-blooded animals (37 degrees) so that they are unable to grow at even moderate temperatures.

Thermophiles are adapted to temperatures above 60 degrees in a variety of ways. Often thermophiles have a high G + C content in their DNA such that the melting point of the DNA (the temperature at which the strands of the double helix separate) is at least as high as the organism's maximum T for growth.

But this is not always the case, and the correlation is far from perfect, so thermophile DNA must be stabilized in these cells by other means.

The membrane fatty acids of thermophilic bacteria are highly saturated allowing their membranes to remain stable and functional at high temperatures. The membranes of hyperthermophiles, virtually all of which are Archaea, are not composed of fatty acids but of repeating subunits of the C5 compound, phytane, a branched, saturated, "isoprenoid" substance, which contributes heavily to the ability of these bacteria to live in superheated environments.

The structural proteins (e.g. ribosomal proteins, transport proteins (permeases) and enzymes of thermophiles and hyperthermophiles are very heat stable compared with their mesophilic counterparts.

The proteins are modified in a number of ways including dehydration and through slight changes in their primary structure, which accounts for their thermal stability.

QUIZZES

1. Choose the nutrient medium for obtaining the separated colonies:
A. Meat-peptone broth
B. Sugar broth
*C. Meat-peptone agar
D. Kitt-Tarozzi medium
E. Alkaline peptone water

2. We can obtain pure culture of anaerobic microorganisms with the help of:
A. Fortner's method
B. Shukevich's method
C. Paster's method
*D. Weinberg’s method
E. Loeffler's method

3. What is the value of a hanging-drop preparation?
A. For study sensitivity to antibiotics
B. For examination of biochemical properties
C. For study morphology of bacteria
*D. For study motility of bacteria
E. For study cultural properties of bacteria

4. Choose obligate anaerobes among these bacteria:
A Neisseria gonorrhoeae, Streptococcus pneumoniae
B. Mycobacterium tuberculosis
*C. Clostridium tetani, Clostridium botulini

D. Bacillus anthracis, Brucella melitensis
E. Shigella dysenteriae, Salmonella typhi

6. The second stage of obtaining the pure culture of aerobes is:
A. Inoculating of a mix (material) on a solid nutrient medium
B. Check of cleanliness of the pure culture and its identification
C. Biochemical and serological identification
*D. Studying of isolated colonies and inoculating on the slant agar
E. Microscopic examination of a material

7. The third stage of obtaining the pure culture of aerobes is:
A. Check of cleanliness of the pure culture and its identification
B. Inoculating of a mix (material) on a solid nutrient medium
*C. Biochemical and serological identification
D. Studying of isolated colonies and inoculating on the slant agar
E. Microscopic examination of a material

8. We can study proteolytic properties of bacteria by detection in tubes of:
A. Carbonic acids and waters
B. Glucose and lactose
C. Carbonic acids and nitrogen
D. Manitolum and methanol
*E. Indol and hydrogen sulphite

9. What microorganism is a sanitary – indicative in studying of quality of running water?
A. Staphylococcus aureus
B. Corynebacterium diphtheriae
C. Streptococcus pneumoniae
*D. Escherichia coli
E. Vibrio cholerae

10. To check up the work of autoclave a laboratory assistant used a chemical substance melting in temperature 119^0 C. What is this substance?
A. Nitrogen
B. Potassium
C. Benzyl
D. Silver
*E. Sulfur

11. While studying inoculations of air taken from chemists a bacteriologist found a sanitary – indicative microorganism. What is this microorganism?
A. Escherichia coli
B. Vibrio cholerae
C. Streptococcus pneumoniae

*D. Staphylococcus aureus
E. Mycobacterium tuberculosis

12. A laboratory assistant sterilized simple nutritious media and physiological solution in an autoclave. What regime of work was used in the autoclave?
*A. 120 degree C (1atm.)
B. 115 degree C (0,5atm.)
C. 100 degree C (0atm.)
D. 134 degree C (2atm.)
E. 148 degree C (3atm.)

13. In inoculation of faeces for nutritious medium both the red colonies with metallic luster and colorless colonies developed. How do we call this nutritious medium?
A. Blood agar
B. Chocolate agar
C. Kitt - Tarrozzi medium
*D. Endo medium
E. Hiss medium

14. To isolate an agent of anaerobic infection a bacteriologist made an inoculation of studied material into liquid nutritious medium covered by oil. How do we call this medium?
A. Endo medium
B. Chocolate agar
C. Hiss medium
D. Blood agar
*E. Kitt – Tarrozzi medium

15. The answer from laboratory indicates the minimal therapeutic dose of antibiotics which the agent is sensitive to. In what method was this dose determined?
A. Membrane filter method
B. Sedimentations method
C. Serological method
*D. Dilution test
E. Diffusion test

16. To determine the agents sensitivity to antibiotics the method was used in which the small filtrating paper disks soaked with antibiotics are brought on the medium inoculated with staphylococci culture. How do we call this method?
A. Sedimentations method
B. Serological method

*C. Diffusion test
D. Membrane filter method
E. Dilution test

17. At the chemists a medical preparation was sterilized by the method of tindalization. What apparatus was used?
A. Hot air oven
B. Membrane filter
C. Autoclave
*D. Water bath
E. Steamer

18. To characterize the qualities of medicinal preparation, a chemical – therapeutical index was introduced. The index was proposed by:
A. Lister
B. Neisser
C. Leeuwenhoek
D. Robert Hook
*E. Ehrlich

19. To isolate an agent, a bacteriologist inoculated by stroke a studied material on dense nutrious medium in a Petri dish divided into four sectors. What method of isolation of pure culture was used?
A. Biological
B. Bacteriological
*C. Mechanical
D. Allergic
E. Serological

20. A bacteriologist reveled in a strain of colon bacillus the ability to work out the colicines. What is this quality provided by?
A. Flagella
B. Mesosomes
C. Spores
D. Ribosomes
*E. Plasmids

21. Staphylococcus is isolated from a discharge of middle ear. A bacteriologist inoculated an isolated culture into a test – tube with citrate rabbit plasma. What enzyme of aggression did he want to reveal?
*A. Coagulase
B. Hemolysin
C. Leucocidin
D. Fibrinolysin
E. Hyaluronidase

22. Pathogenic staphylococcus was isolated from a suppurative wound of a patient, its sensitivity to antibiotics was determined. Penicillin caused a zone of growth retention – 8mm; oxacillin – 9mm; ampicillin – 10mm; gentamicin – 22mm; lincomycin – 11mm. What antibiotic should be chosen for treatment of the patient?
A. Penicillin
*B. Gentamicin
C. Ampicillin
D. Lincomycin
E. Oxacillin

23. While determining the sensitivity staphylococcus to antibiotics, the following results were obtained: diameter of zone of growth retention of penicillin – 7mm; oxacillin – 8mm; ampicillin – 26mm; gentamicin – 22mm; lincomycin – 15mm. What method of investigation was used?
A. Bacteriological
B. Serological
*C. Diffusion test
D. Chemical test
E. Dilution test

24. Nutrious media containing substances destroying in temperature more than 100 degree C (urea, carbohydrates, proteins) should be sterilized for bacteriological examination. What method of sterilization can be used in this case?
A. Sterilization by filtration
B. Ultraviolet radiations
*C. Tindalization
D. Autoclavation
E. Infra red radiation

25. To obtain exotoxin, a toxigenic microorganism is inoculated into liquid nutrious medium. After toxins accumulation in the medium, microbe cells are removed from it. What device is used in this case?
A. Autoclave
B. Boiling
*C. Membrane filter
D. Hot air oven
E. Steamer

26. To confirm clinical diagnosis of an infectious disease, a laboratory study is carried out. What method of isolation of pure culture of microorganisms is used?
A. Serological
B. Biological

C. Bacterioscopical
*D. Bacteriological
E. Allergic

27. Corresponding nutrious media are used in bacteriological laboratories to isolate a pure culture and identify the isolated microorganism. What medium is used to isolate and identify staphylococcus?
A. Endo medium
B. Hiss medium
*C. Blood agar
D. Chocolate medium
E. MPA

28. At present the description of ten kinds of staphylococci is represented. They are revealed in people, domestic and animals. But only one kind is of great importance in the infectious pathology of man. How do we call this kind?
A. Staphylococcus epidermidis
B. Staphylococcus saprophyticus
C. Staphylococcus albus
*D. Staphylococcus aureus
E. Staphylococcus citreus

29. When studying the microflora of air in chemists, a pure culture of a microorganism was isolated. The microorganism grows and develops if there is not less than 20 per cent oxygen in atmosphere. What group does an agent belong to according to the type of breathing?
A. Microaerophiles
B. Facultative anaerobes
C. Obligate anaerobes
*D. Obligate aerobes
E. Aerotolerant microorganisms

30. The consignment of battles with glucose solution for parenteral use in prepared in a chemists. What way of sterilization is necessary to use?
A. Sterilization by filtration
*B. Autoclavation
C. Tindalization
D. Hot air over
E. Ultraviolet radiation

31. The formation of acquired stability to antibiotics occurs not only by the way of selection of spontaneous antibiotic stable mutants of the bacteria, but also due to spreadening among microorganisms particular factors which are called:

A. F - pili
*B. R - plasmid
C. A - protein
D. Spore
E. Cell wall

32. Sanitary – indicative microorganisms are chosen for characteristics of bacterial contamination of soil by man and animal. What microorganism present in soil is an index of long fecal contamination?
A. Staphylococcus
B. Enterococcus
*C. Clostridia
D. Neisseria
E. Mycobacteria

33. Chemical substances destroying microorganisms in environment are called disinfecting. What concentration of ethyl alcohol is most effective to manifest antimicrobic effect?
A. 100 degree
*B. 70 degree
C. 80 degree
D. 40 degree
E. 50 degree

34. Which of the following is an alkylation agent used as a disinfectant?
Merbromin
Iodouridine
Silver nitrate
Phenol
*E.Glutaraldehyde

35. What antimicrobial agent displays specificity for anaerobic microorganisms?
A.*Metronidazole
B. Polymyxin
C.Vancomycin
D.Gentamicin
E. Oxacillin

36. What is the single most frequent etiologic agent of ascending urinary tract infection?
A. Klebsiella pneumoniae
B. Serratia marcescens
C. Citrobacter freundii
D. Enterobacter cloacae

E. *Escherichia coli

37. Which bacteria are most numerous in the human gastrointestinal tract?
A. Escherichia species
B. *Bacteroides species
C. Pseudomonas species
D. Enterobacter species
E. Proteus species

38. Staphylococcus aureus is isolated from a pus. A bacteriologist plated an isolated culture on blood agar. What enzyme of aggression did he want to reveal?
A. Fibrinolysin
B. Hyaluronidase
C. *Hemolysin
D. Leucocidin
E. Endotoxin

39. Escherichia coli is isolated from an intestinal discharge. A bacteriologist reveled in a strain the ability to fermentative the lactose. What nutritious medium he was used?
A. Hiss medium
B. *Endo medium
C. MPW
D. Blood agar
E. MPA

40. For the identification of the microorganisms it is necessary to determine their proteolytic activity. What nutritious medium can be used for it?
A. *Meat peptone water
B. Meat peptone agar
C. Blood medium
D. Kitt – Tarrozzi medium
E. Endo medium

41. The operation theatre was sterilized. What physical method of sterilization can be used for it?
A. Autoclavation
B. Tindalization
C. *Ultraviolet radiation
D. Filtration
E. Hot air oven

Recommended reading list

Main literature

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4. Jawetz, Melnik & Adelberg's Medical Microbiology [Текст] : учебное пособие. - 22 Edition. - New York : Lange Medical Books/McGraw-Hill, 2001. - 695 p.
5. Medical Microbiology : textbook / D. Greenwood [et al.]. - 17th ed. - Toronto : Churchill Livingstone, 2007. - 738 p.

Further Reading

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2. Microbiology. A human perspective / M. T. Nester, E. V. Nester, C. E.Roberts. - 1995.
3. Levenson W. E. Medical microbiology and immunology / W. E. Levenson, E. Javetz. – Norwalk, 1994,
4. Krivoshein Yu. S. Handbook on microbiology / Yu. S. Krivoshein– Moscow : Mir Publishers,.1989

Informational resourses:

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2. http://www.yteach.co.uk/index.php/search/results/AQA_GCSE_Science_A_(4461)_Biology,3,0,7033;7230,0,25,1,wa,1.html

3. American Society for Microbiology — http:// asm.org.;
4. http://journals.asm.org; (American Society for Microbiology) — http:// asm.org.;
5. http://www.news-medical.net/health/Virus-Microbiology-(Russian).aspx;
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