**LESSON 11.**

**Cultivation method. Isolation of the pure culture of aerobic and anaerobic bacteria (II and III days). Cultural character of bacteria. Identification of bacteria by enzymatic activity. Modern identification methods**

**LESSON PLAN:**

* The concepts of "cultivation of microorganisms", "culture", "clone", "colony" and "strain".
* Products of vital activity of microorganisms: enzymes and pigments, aromatic substances and their importance.
* Cultural properties of bacteria - macroscopic and microscopic examination of colonies (size, shape, color, transparency, consistency, location, surface, edges and structure).
* Counting of colonies, the essence of *colony formation unit* (CFU) in various pathological materials.
* Classification of bacterial enzymes.
* The enzymes roles in biochemical properties and identification of bacteria.
* The enzymes that break down carbohydrates and their determination (Hiss and Kligler medium).
* Proteolytic enzymes and their determination (development in gelatin, serum and milk, determination of indole, ammonia and hydrogen sulfide) and oxidation-reduction enzymes (oxidase, catalase, decarboxylase).
* Aggression enzymes and their determination (hyaluronidase, lecithinase, fibrinolysin, plasmacoagulase).
* Modern identification methods (microtest systems, Vitek analyzer, etc.).

**II stage of cultural method**

* Begins with evoluation of cultural features of bacteria
* Inoculated Petri dishes are taken from incubator and cultural features are examined
* Consistent dilution of microorganisms inoculated by Driqalski method on nutrient media is observed. Isolated colonies are seen commonly on 2nd and 3rd plates.
* During 4-sector inoculation pattern of bacteria growth depends on number of microorganisms in specimen. Isolated colonies are observed on last inoculated sector.
* One colony is believed to originate from one bacterial cell
* The methods designed to obtain pure culture are based on growth of isolated colonies on surface or bottom of solid media
* At second stage isolated colonies are passaged to other Petri dishes and incubated for 1-2 days.
* Culture – population of bacteria formed at optimal growth conditions
* Colony - population formed by one bacterial cell on the surface of medium.
* Pure microorganism culture – a population of one species on surface of solid medium
* Strain – pure culture of one species obtained from different sources or from the same source during different time.
* Cultural features are special for each species or genus and can be used in identification of microorganisms.
* For this purpose growth features of microorganisms in solid and liquid media are examined.
* Bacteria form *colonies* on solid media.
* Population formed by one bacterial cell inside or on surface of solid media is called *colony*.
* Features of colonies:
* Size
* Shape
* Colour
* Texture
* Elevation
* Margins
* Large (more 4-5 mm)
* Medium (2-4 mm)
* Small (1-2 mm)
* Punctiform (less 1 mm)
* Some bacteria produce pigments during growth on medium
* Transparency levels:

-transparent

-translucent (semi-transparent)

-opaque

III stage of pure culture

* AT 3rd stage pure culture is checked for “pureness”
* Smear from slant culture of bacteria is prepared, stained by Gram method and examined under the microscope. Presence of only one morphological type of bacteria proves “pureness” of culture
* Further, biochemical features of culture are tested
* At the final stage of bacteriological method identification of microorganism, in other words detection of its genus and species name, is performed.
* *Identification* is performed based on cultural, tinctorial, morphological, antigenic, fermentative etc. features.
* Biochemical features of bacteria are investigated via enzyme and metabolites testing.
* Enzymatic features are the main taxonomic value in identification.
* Sacharolytic, proteolitic and other enzymes are tested.
* Microorganisms produce variety of genetically determined enzymes. 6 class enzymes perform all metabolic reactions in microbial celli:
1. Oxydo-reductases(catalysts of oxydation-reduction reactins),
2. transferases (transfer of atoms between different molecules),
3. Hydrolases (destruction of protein, carbohydrat, lipid molecules using water molecules),
4. ligases (connection of 2 molecules by new chemical bond),
5. liases (qeyri-hidrolitik yolla kimyəvi qrupları ayırır),
6. isomerases (metabolism of carbohydrates).
* Endoenzymes are located inside the cell, exoenzymes are secreted in environment.
* *Endoenzymes* work inside the cell*, exoenzymes* are secreted outside the cell and act on macromolecules, thus, facilitating their transport inside the cell.
* *Constitutive and inducible enzymes*
* *Metabolic enzymes*– oxydo-reductases, transfеrases, liases, ligases, hydrolases and isomerases
* *Agression, pathogenicity enzymes* – hyaluronidase, neuraminidase, lecytinase etc.
* Enzymatic features are important taxonomic features used in identification.
* For identification *sacharolytic*, *proteolytic* etc. enzymes are tested.
* Hyss “colorful row” can be used for this purpose. This row includes tubes with solid and semisolid nutrient media. Each test tube contains carbohydrate. All tubes contain indicator changing its colour related to pH. Thus, change of color in test tube means that microorganism is able to utilize carbohydrate in test tube.
* During carbohydrate utilization some bacteria produce acid, others both acid and gas.
* Special glass tube inside the test tube serves for gas detection in tube. Produced gas is captured inside this glass tube and can be observed by examiner.
* In semisolid media gas bubbles can be seen
* *Sacharolytic features* are tested by inoculation of pure culture of bacteria obtained at 3rd day of bacteriogical examination in test tubes, incubation at 37°C for 18-24 hours.
* Bacteria utilizing carbohydrates with production of acid change color of medium, those which produce acid and gas change color and in addition form gas bubbles. In absence of fermentation no change of color is observed. Different bacteria form different pattern of carbohydrate fermentation – thus forming “colorful” row.
* Short and long colorful” row can be used.
* Short colorful” row consists of mono- və disacharides (glucose, lactose,sacharose, maltose) containing tubes with Hyss media.
* long colorful” row includes above mentioned media with additional tubes with various monosacharides(arabinose, xylose, ramnose, galactose), polisacharides(inulin, starch, glykogen), alcohols(glycerin etc.)
* All tubes contain indicator - Andrede reagent.
* Proteolytic features are investigated through detection of ability of bacteria to utilize gelatin and produce ammonium, indole, hydrogen sulfide etc.
* *Proteolytic enzymes are detected through by inoculation of peptone and*  10-20%- g*elatine with bacterial culture. Inoculated media are incubated for a few days at* 20-220C.
* Proteolytic enzymes break down gelatin with formation of characteristic patterns: “nail” and “inverted pine”.
* Peptone water breakdown in 370C is accompanied with production of ammonium, indole, hydrogen sulfide etc. which can be detected by adding special reagents.

Indole detection

* *Ehrlich method*: 2-3 ml of ether is added to the test tube containing the bacterial culture, mixed and a few drops of *Ehrlich* reagent (alcohol solution with para-dimethyl-amide-benzaldehyde hydrochloric acid) is added. Production of indole is accompanied with pink color.
* *Morel method*: A thin strip of oxalic acid-impregnated filter paper is fixed above the nutrient medium. The change of lower part of strip to pink color indicates the formation of indole.
* Bacteria is inoculated in liquid tryptophane containing media and incubated at 37°C for 1 day.
* 1-2 drops of Kovac reagent (paradimethylaminobenzaldehyde) is added.
* Formation of red colored ring in reaction area indicates presence of indole.

Hydrogen sulfide detection

* Lead-acetate containing paper strip is fixed above bacteria culture and incubated. Change of strip color to black indicates formation of hydrogen sulfide.
* Another method is based on inoculation (stubbing) of culture in medium containing ferrum sulfate, sodium sulfate, sodium tiosulfate. H2S formation changes color of medium to black.

Ammonium detection

* Litmus indicator strip is placed above culture of microorganisms.
* Change of indicator color to blue after incubation indicates presence of ammonium.

Catalase detection

* 1 drop of 1-3% Hydrogen peroxide is placed on glass slide and mixed with culture of bacteria. In presence of catalase oxygen and water is formed from hydrogen peroxide is .
* Visually observer can see formation of gas bubbles.

Oxidase

* Principle. Some bacteria have cytochromoxydases functioning as hydrogen transporters to oxygen.
* Oxidase converts colorless dye phenyldamine dihydrochloride (an artificial oxygen acceptor) to blue indophenol.
* Procedure. Culture of bacteria is placed on paper strip or disk containing indicator using loop or applicator.
* Positive reaction is accompanied with formation of blue or violet color.

Differential media

* Differential media help to differentiate or even identify microorganisms.
* Differentiation is based on enzymatic features of microorganisms.
* Examples of differenetial media: Еndo, *MacConkey,* eosin methylene blue (EMB).

Еndo

Contains 1% lactose and indicator (basic fuchsin decolorized with sodium thiosulfite).

The color of Endo medium is pink.

Bacteria utilizing lactose form red colonies with metallic (due to formation of acid from lactose),

Lactose non-fermenters form colorless colonies

Kligler

Contains: 1% lactose, 0,1% glucose, Na-tiosulfat, Fe-sulfat, İndikator

Prepared medium in test tubes in form of slants.

Inoculation– stubbing bottom and spread on slant surface

* Only glucose fermentation results in color change of bottom
* When both glucose and lactose utilized whole agar color (bottom and slant colors change)
* H2S formation is accompanied with black color (salmonella, proteus)

TSI (triple sugar iron) agar

* Contains:
* 1% lactose
* 1% sacharose
* 0,1% glucose (if it breaks down, the agar column turns yellow)
* Fe-sulfate – detects formation of H2S (black color)
* pH-indicator phenol red

İMVİC test(4 test )

* İndole
* Methyl red
* Voges-Proskauer
* Citrate

API sistem *(Application programming interface)*

… Before APİ test implementation pure culture must be obtained and preliminary test should be performed

test 1 : Gram stained smear microscopy result (Gram-, Gram+, rod, cocci etc.)

test 2 : Respiratory enzyme tests🡪 oxydase, catalase

Novel automated system for microorganism identification

* *Vitek 2 Compact* anlyzer– full automated system identifying microorganism and antibiotic susceptibility testing in 5-8 hours.
* Identification is performed based on automatic testing of biochemical features of microorganism. In case if full identification is impossible, the system gives probability of microorganism name in percentage.
* All automated systems require pure culture of microorganism to be identified.
* Obtained pure culture is placed in cassette of analyzer and results are obtained after some tome which required for incubation and interpretation of performed tests.
* Analysis gives genus and species name of microorganism and antibiotic susceptibility testing.
* Moreover, analyzer give information about minimum inhibitory concentration (MIC) of antimicrobial drug and genetic mechanisms of resistance.

Matrix activated laser desorbtion/ionization (MALDİ-TOF)

Automate system using mass spectrometry

Principle

Physical detection of cell proteins via mass spectrometry

+ obtained spectrum is compared with database

* MALDI-tof mass spectrometry (Matrix Assisted Laser Desorption/Ionization)
* As a result of the interaction of laser rays with the analyzed substance in the matrix, the substance is ionized
* Non-volatile high-molecular compounds (peptides, proteins, oligonucleotides, carbohydrates) are determined.
* Japanese engineer CT Shimadzu received the Nobel Prize in 2002 for creating the MALDI-tof device.

*Biomerieux VITEK-2 - bacteriological analyzer*

Vitek-2 Compact analyzer is an automatic system.

 Identification of microorganisms

Sensitivity to antimicrobial drugs is determined (within 1 day)

It consists of plastic cards with 64 depressions

 Gram negative bacteria

Gram positive bacteria

Yeast mushrooms

Anaerobic bacteria, neisseria, hemophilic bacteria

Highly virulent microorganisms include: Brucella melitensis, Burkholderia pseudomallei, Francisella tularensis, Burkholderia mallei, Escherichia coli O157, Vibrio cholerae, Yersinia pestis.

The time to get the result is 6-8 hours.