AZERBAIJAN MEDICAL UNIVERSITY
DEPARTMENT OF MEDICAL MICROBIOLOGY and IMMUNOLOGY

Lesson 11.

Bacteriological 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

FACULTY: General Medicine
SUBJECT: Medical microbiology - 1

Discussed questions:

- 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.).

Purpose of the lesson:

- To acquaint students with the purpose, stages and cultural characteristics of obtaining pure cultures of bacteria. To inform them about the life activity products of bacteria - enzymes and pigments, their role in the differentiation of bacteria. To acquaint students with modern identification methods of bacteria.

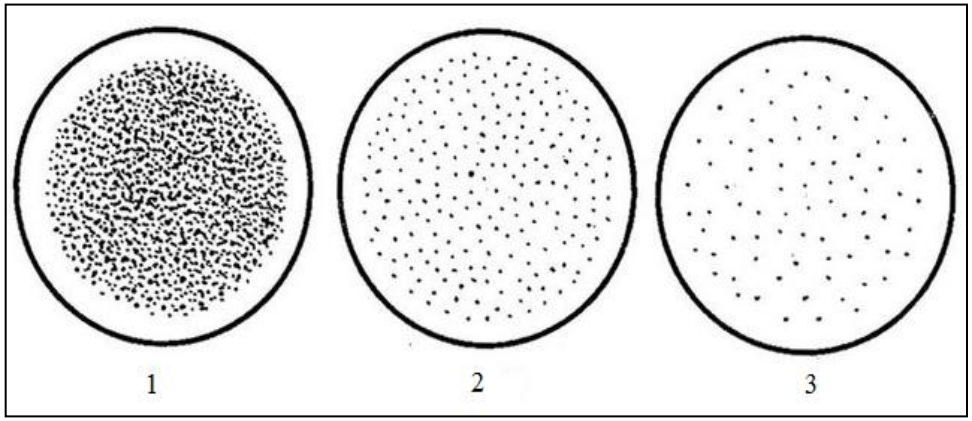
II stage of cultural method

- 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 2^{nd} and 3^{rd} plates.
- During 4-sector inoculation pattern of bacteria growth depends on number of microorganisms in specimen. Isolated colonies are observed on last inoculated sector.

II stage of cultural method

- 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.

Isolated colonies on the surface of solid nutrient media



Cultural features

- 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

- 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.

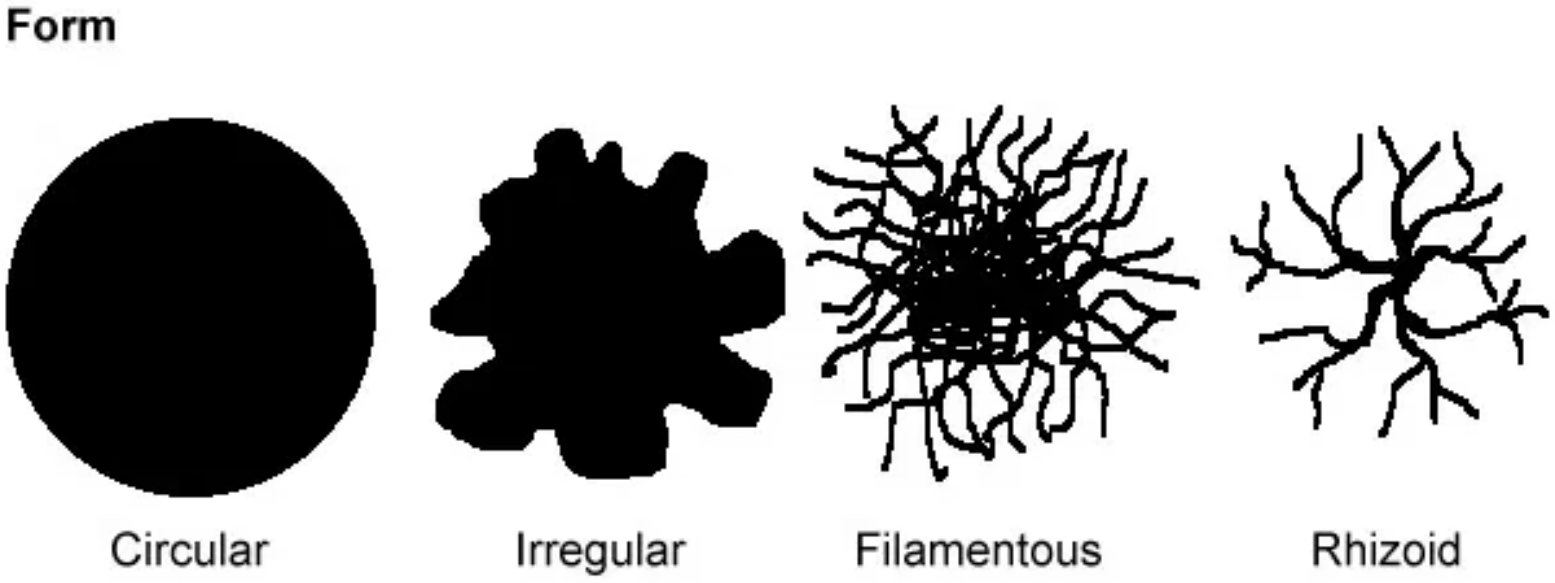
Cultural features on solid media

- Bacteria form *colonies* on solid media.
- Population formed by one bacterial cell inside or on surface of solid media is called *colony*.

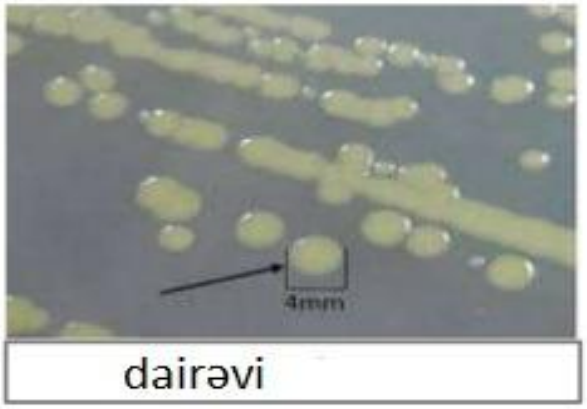
Colony morphology

- Features of colonies:
- Size
- Shape
- Colour
- Texture
- Elevation
- Margins

Different shapes of colonies on agar surface



Colony shapes



dairəvi

nöqtəşəkilli

rizoid

- **Margin of bacterial colony**

Entire (smooth)

Undulate (wavy)

Lobate

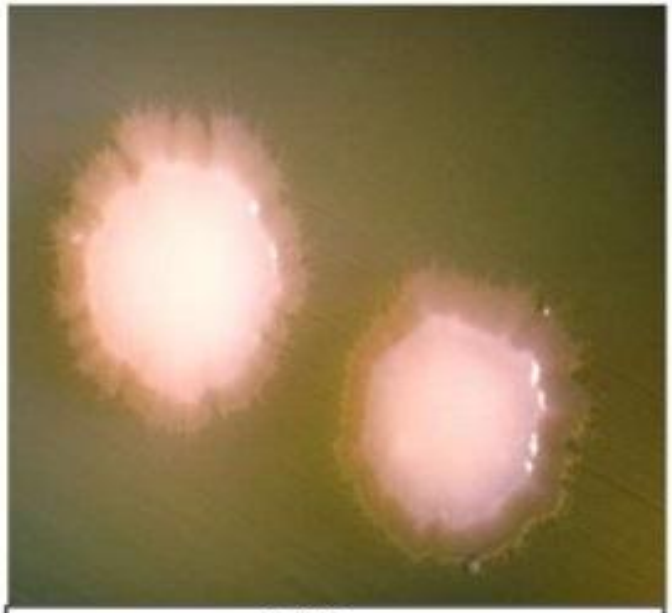
Filamentous

Curled (concentric)

Scalloped

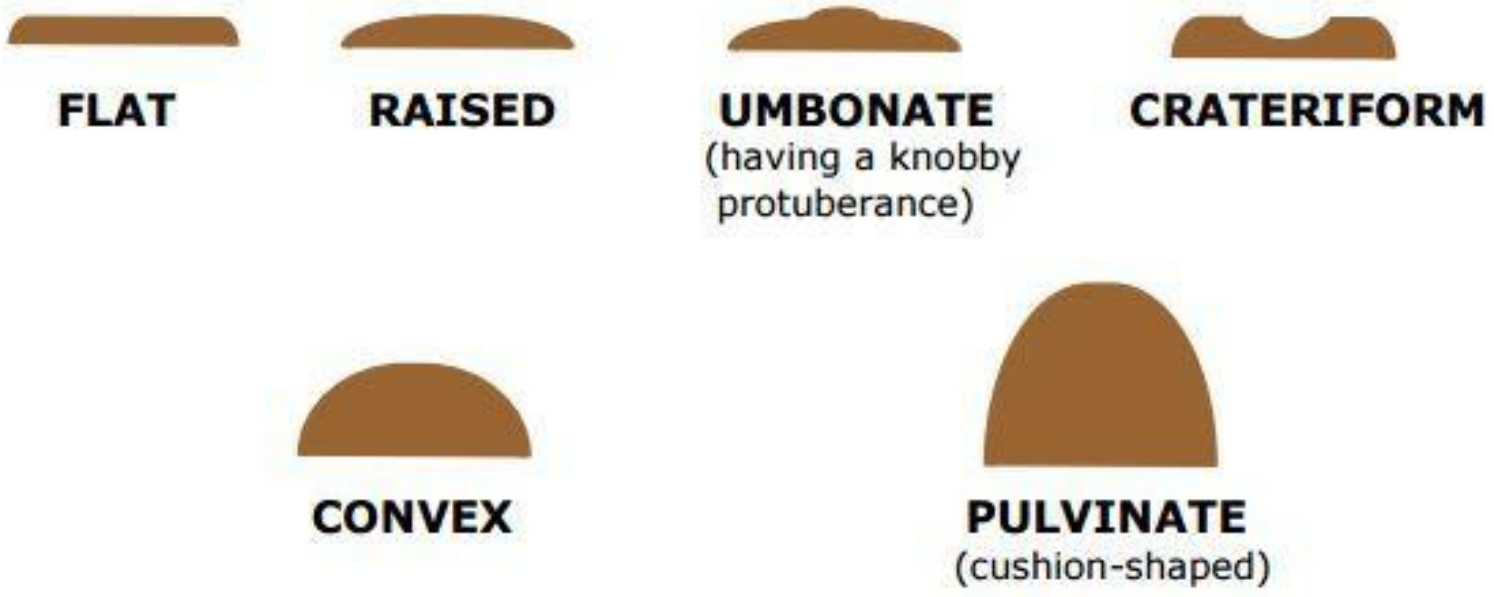
Colony margins

dalğavari



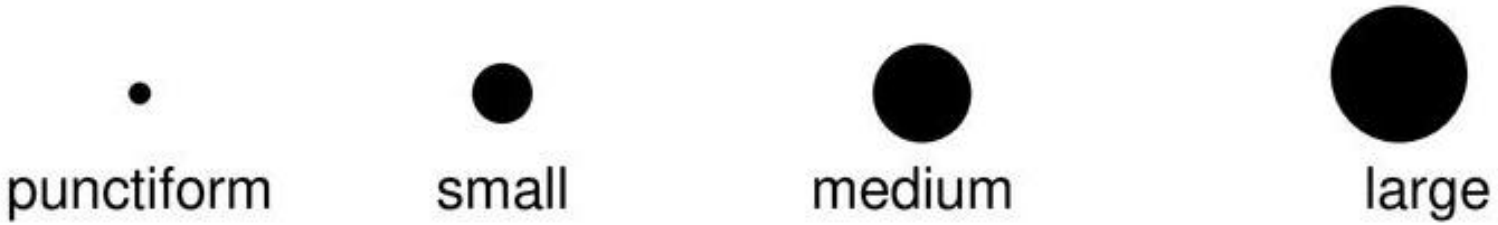
sapşəkilli

Elevation types of colonies on surface of medium

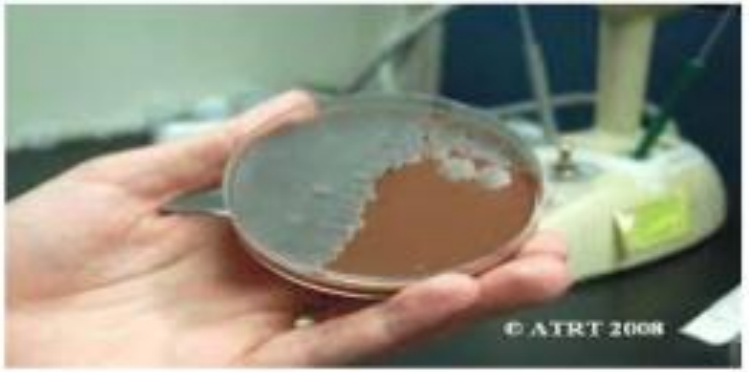


Size of colonies

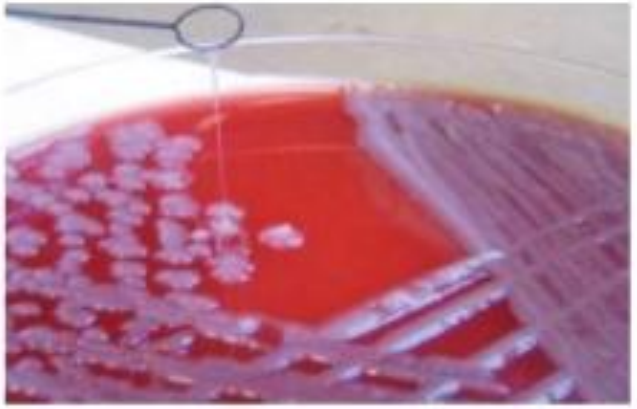
- Large (more 4-5 mm)
- Medium (2-4 mm)
- Small (1-2 mm)
- Punctiform (less 1 mm)



- **Texture of bacterial colony**



- Dry
- Moist
- Viscid (stick to loop)
- Mucoid (mucus-like)



Colony colour

- Some bacteria produce pigments during growth on medium

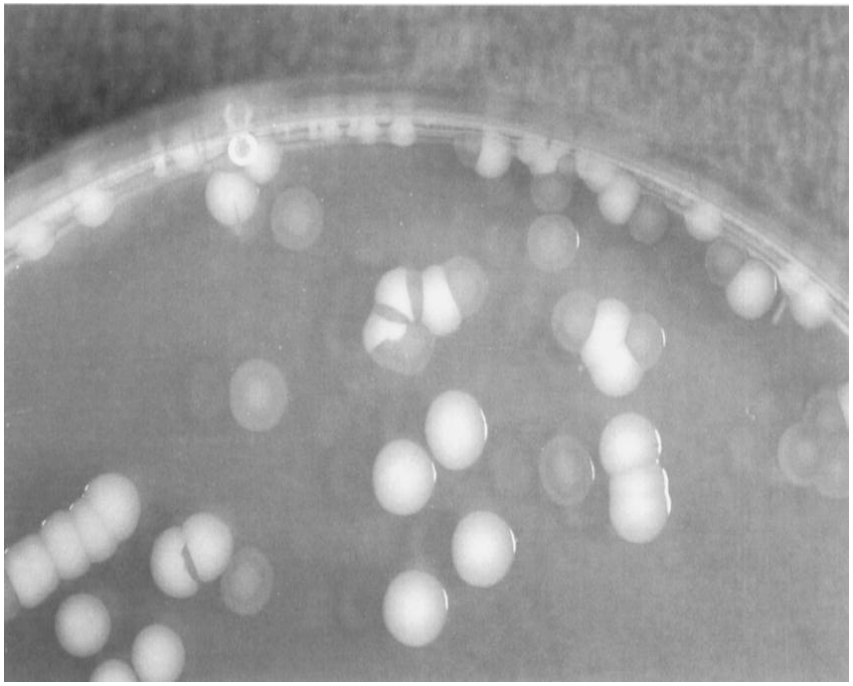
Colony transparency

- Transparency levels:

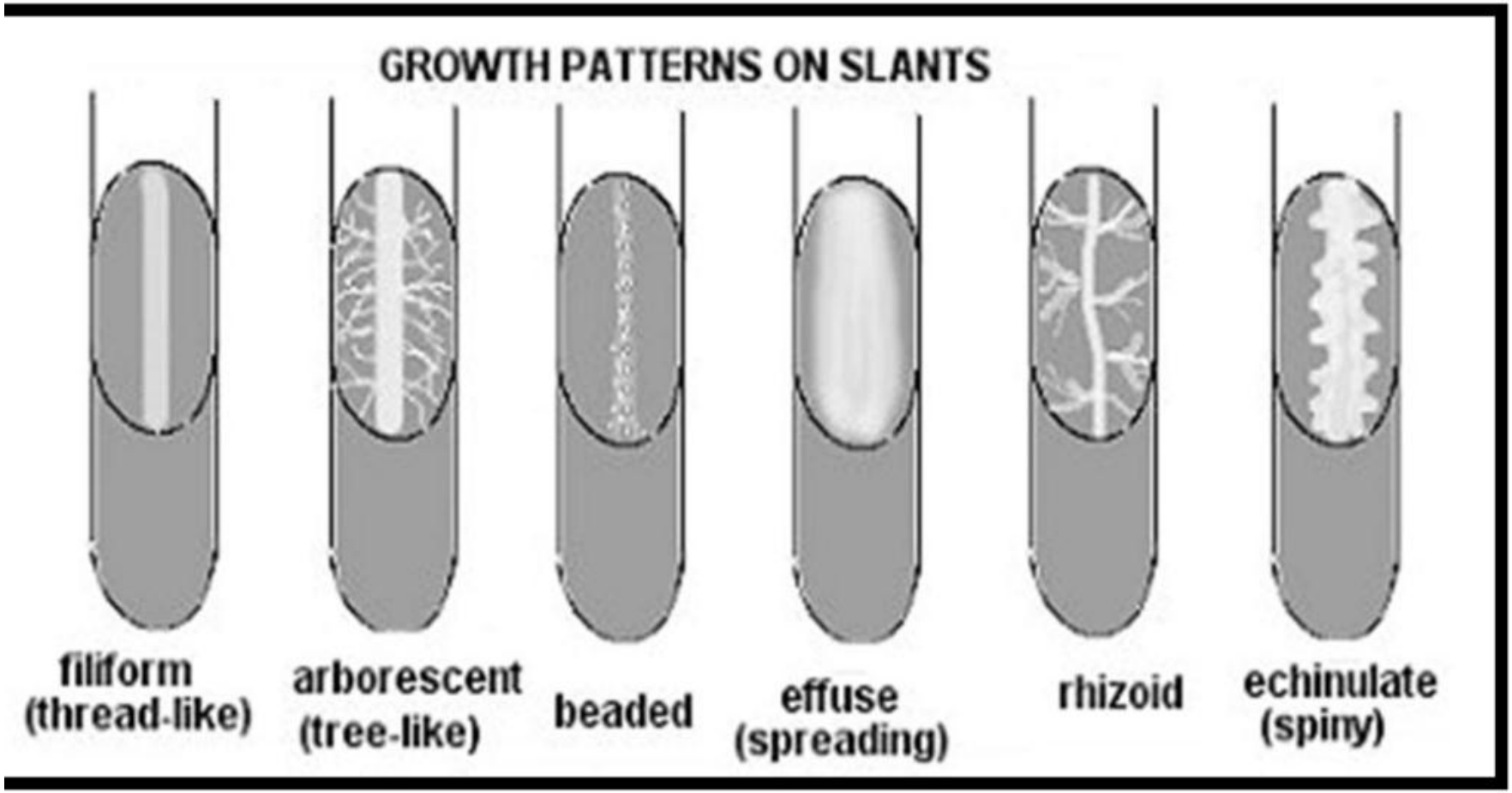
-transparent

-translucent (semi-transparent)

-opaque



PATTERNS OF GROWTH ON A SLANT



Cultural features in liquid media

Colony calculation

Wolf-Huggel chamber is used when colony number is high. This chamber is divided in squares. Petri dish is placed under the chamber and colonies in 10 large squares are counted (1 $cm^{2)\cdot}$. Total count of colonies in one square is calculated by equation:

$$X=\pi r^2 \times 1cm^2 \qquad \pi=3,14$$

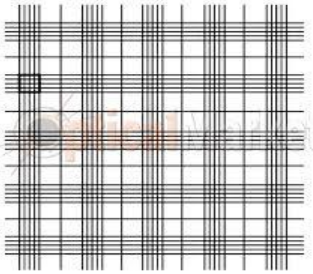
r-Petri dish radius= 5cm

If in 1square= 10 colonies, Then

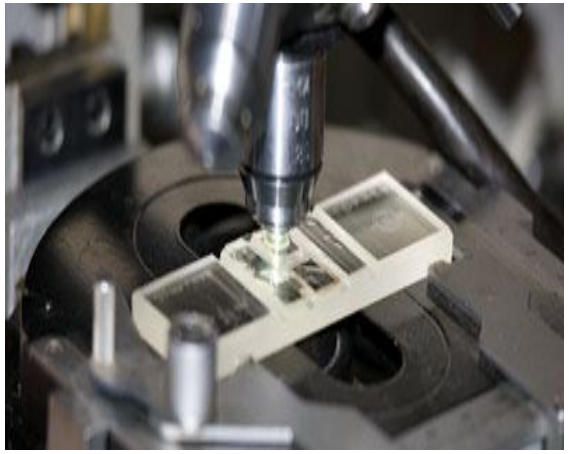
$$X=3,14x5^2 \times 10 = 785$$

Total count of cells in 1 ml of liquid material

1.“Counting chambers” - (Neubauer, Thoma, Goryayev) measurement by microscope
2.Counters Electron haemocytometer - Coulter counter nephelometry (spectrophotometry)
3.Membrane filter method



Goryayev



Measurement of total cell count

Indirect measurement of cell count is based on evaluation of turbidity of bacterial suspension by turbidity standards.

Measured suspension is compared with standard suspension. McFarland is the commonest standard used:

- 1%-sulfate acid
- 1.175%-li BaCl

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.

Stages of cultural method

4th stage:

Stage1 : Sampling

Stage 2 : Cultivation

Stage 3 : Identification

Stage 4 : Result
(genus and species name, antimicrobial susceptibility testing)

Biochemical features of bacteria

- 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.

Microbial enzymes

- 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.

Microbial enzymes

- ***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, transferases, liases, ligases, hydrolases and isomerases
- ***Agression, pathogenicity enzymes*** – hyaluronidase, neuraminidase, lecytinase etc.

Investigation of enzymatic activity

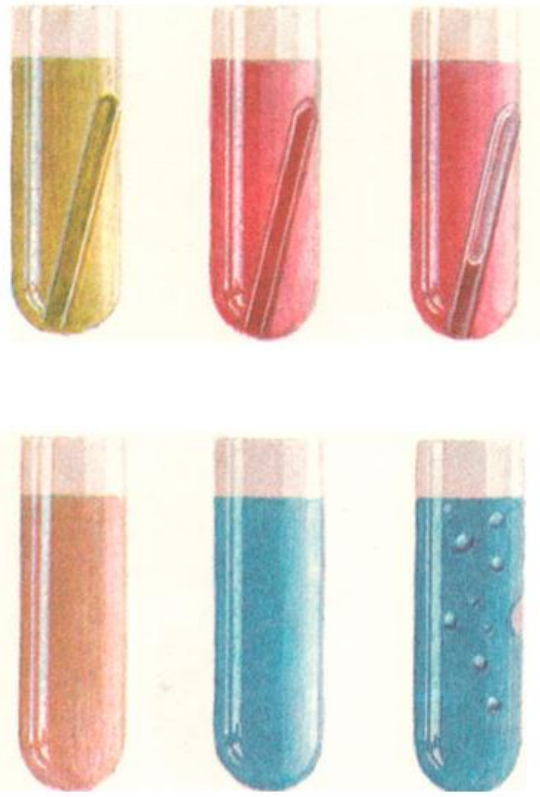
- Enzymatic features are important taxonomic features used in identification.
- For identification *sacharolytic*, *proteolytic* etc. enzymes are tested.

Investigation of ability to utilize carbohydrates (sacharolytic activity)

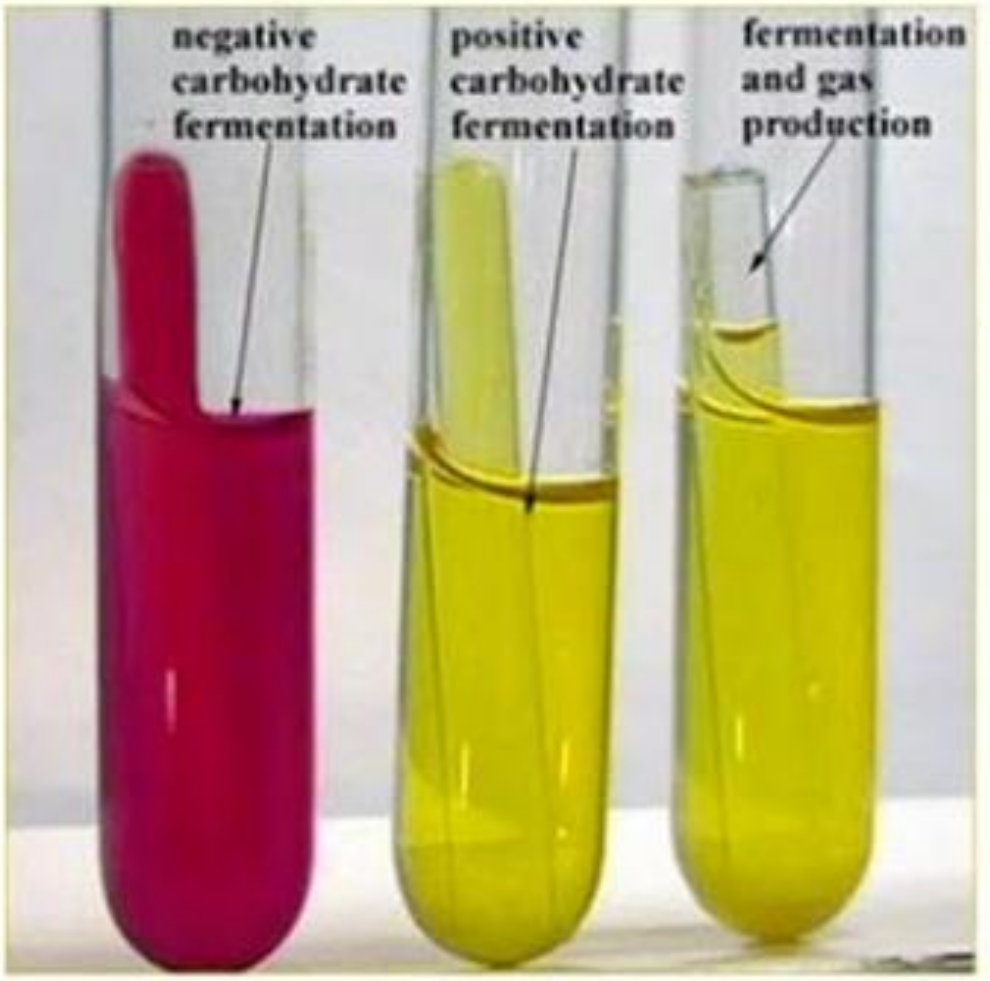
- Hyss “colorful row” can be used for this purpose. This row includes tubes with solid and semisolid nutrient media. Each test tube contains carbohydrate. All tubes contain indicator changing its colour related to pH. Thus, change of color in test tube means that microorganism is able to utilize carbohydrate in test tube.

Hyss “colorful” row

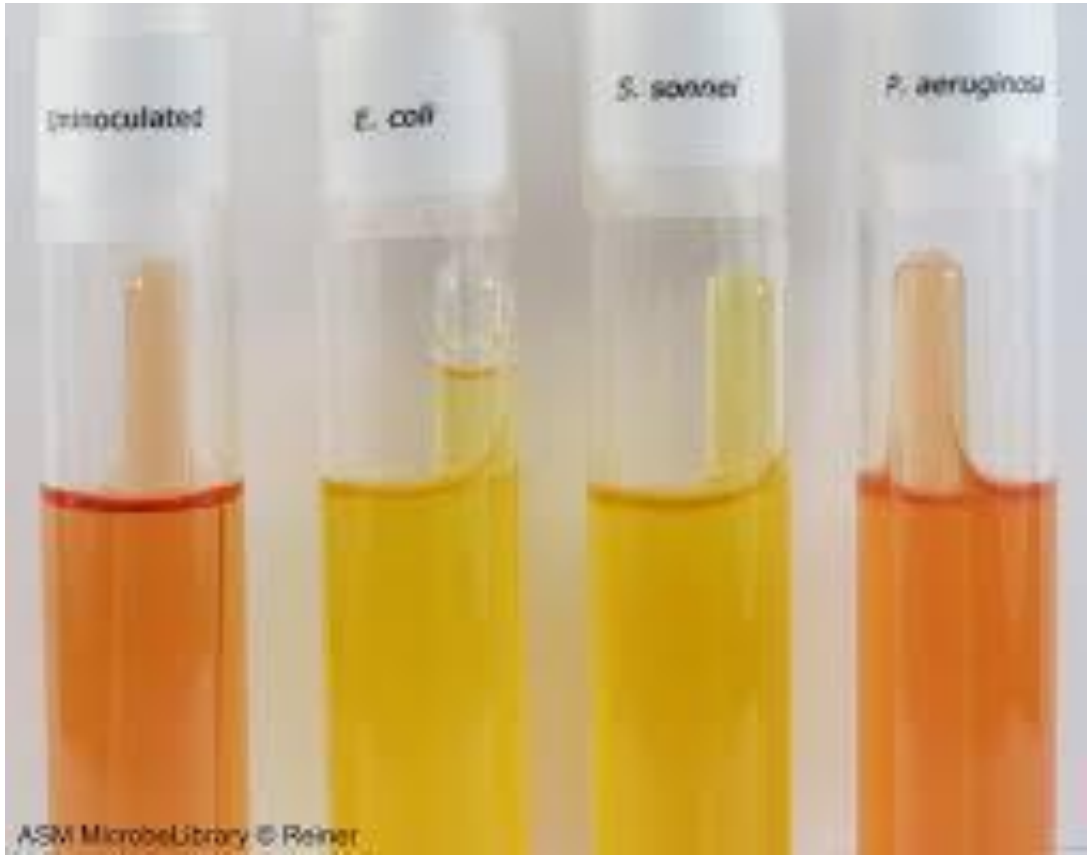
- During carbohydrate utilization some bacteria produce acid, others both acid and gas.
- Special glass tube inside the test tube serves for gas detection in tube. Produced gas is captured inside this glass tube and can be observed by examiner.
- In semisolid media gas bubbles can be seen



Hyss “colorful” row



Hyss “colorful” row



E.coli - breaks down carbohydrates to produce acid and gas.
S.sonnei - breaks down glucose to acid without producing gas.
P.aeruginosa - does not ferment carbohydrates.

Hyss “colorful” row

- ***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.

Hyss “colorful” row

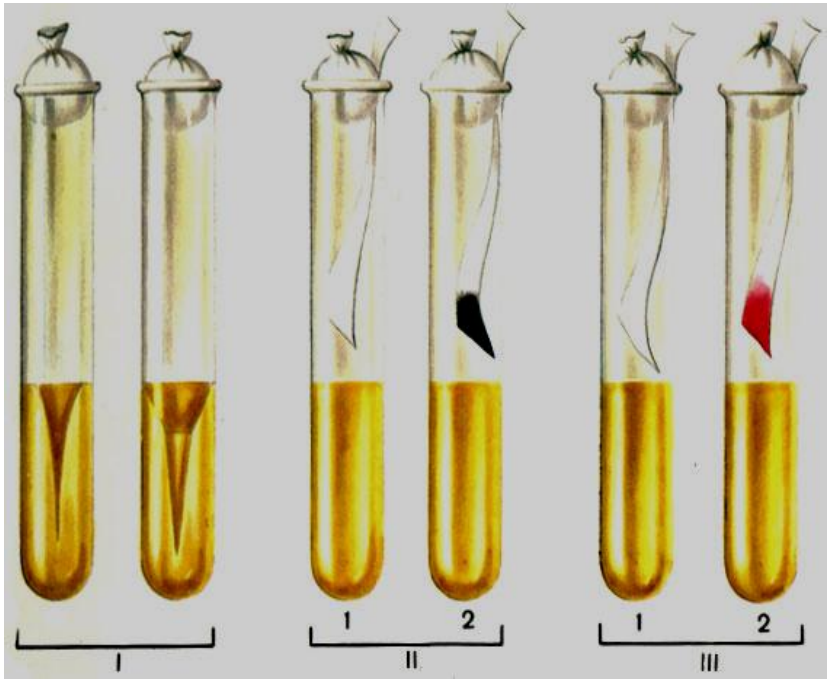
- Short and long colorful” row can be used.
- **Short colorful” row** consists of mono- və disacharides (glucose, lactose,sacharose, maltose) containing tubes with Hyss media.
- **long colorful” row** includes above mentioned media with additional tubes with various monosacharides(arabinose, xylose, ramnose, galactose), polisacharides(inulin, starch, glykogen), alcohols(glycerin etc.)
- All tubes contain indicator - Andrede reagent.

Investigation of ability to utilize proteins (proteolytic features)

Proteolytic features are investigated through detection of ability of bacteria to utilize gelatin and produce ammonium, indole, hydrogen sulfide etc.

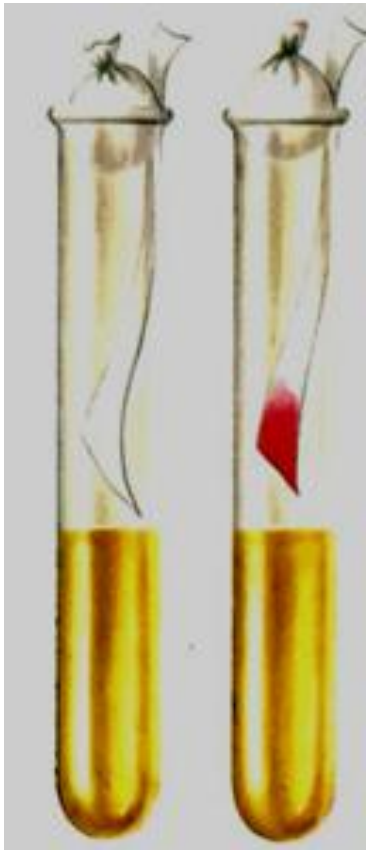
Proteolytic enzymes detection

- ***Proteolytic enzymes*** *are detected through by inoculation of peptone and* 10-20%-*gelatine with bacterial culture. Inoculated media are incubated for a few days at* 20-22^{0}C.
- Proteolytic enzymes break down gelatin with formation of characteristic patterns: “nail” and “inverted pine”.
- Peptone water breakdown in 37^{0}C 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.



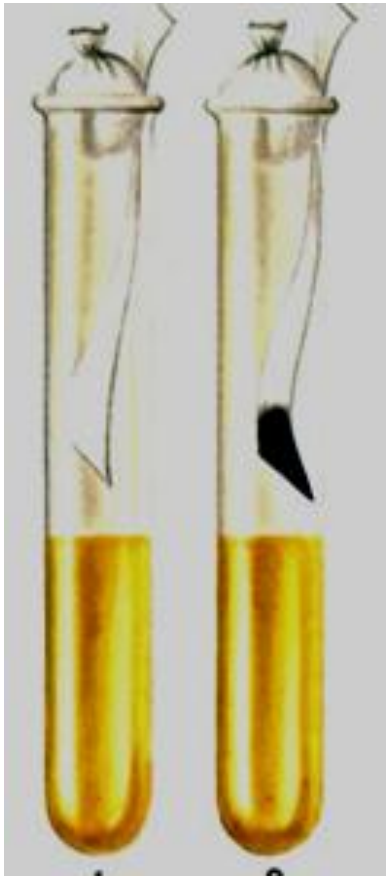
Kovac reagent for Indole detection

- 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.

1. ***Kontrol***
2. ***Escherichiacoli(ATCC25922)***
3. ***Enterobacter aerogenes (ATCC 13048)***

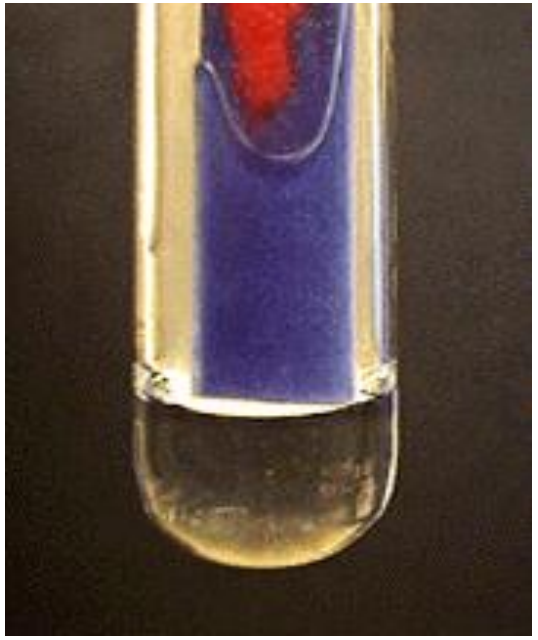
Hydrogen sulfide detection

- 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. H_2S formation changes color of medium to black.



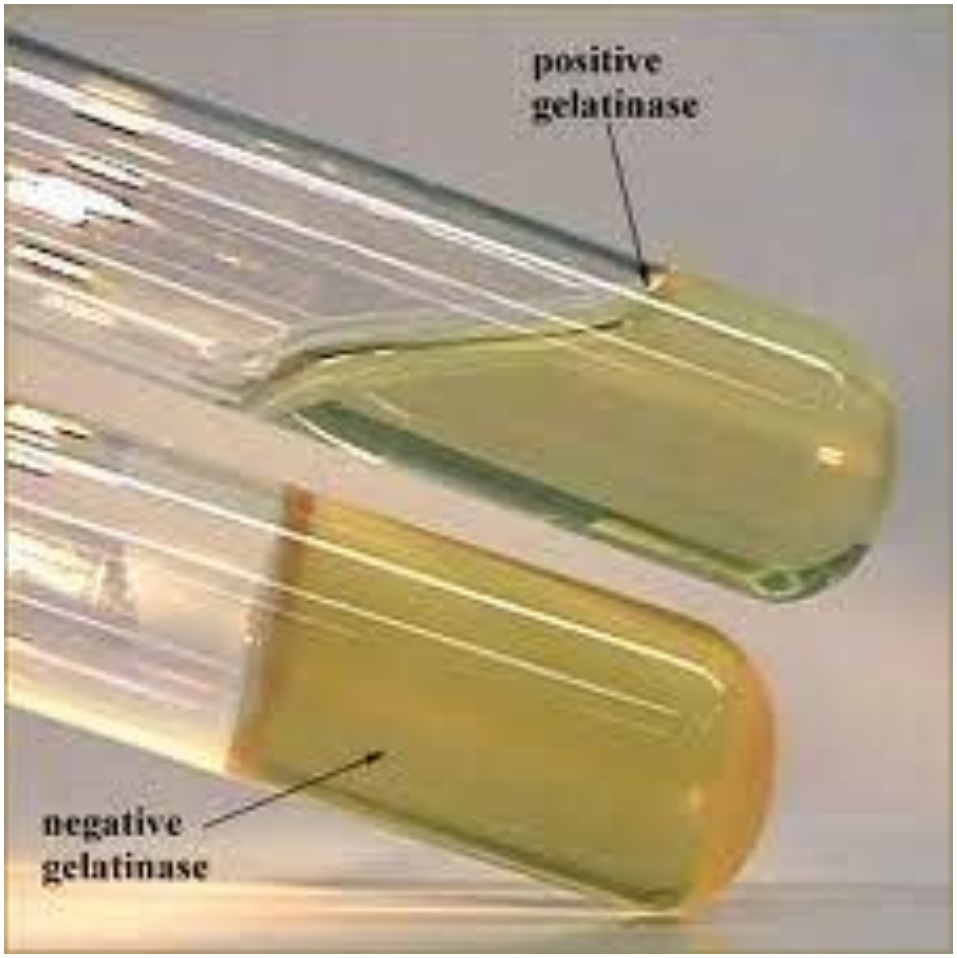
Ammonium detection

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



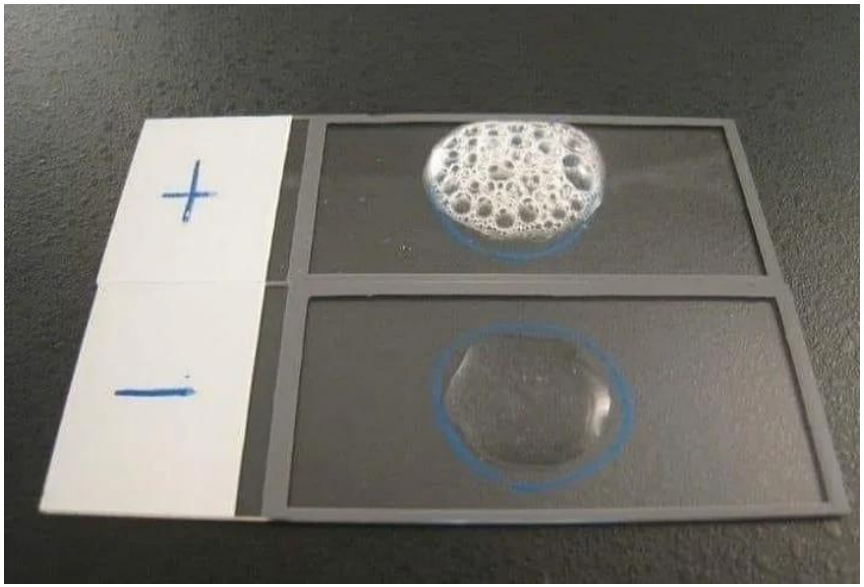
Positive ammonium test

Gelatin testi



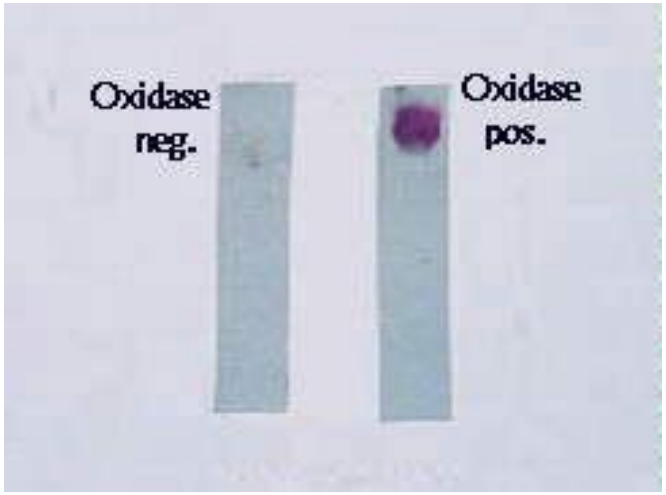
Catalase detection

- 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.



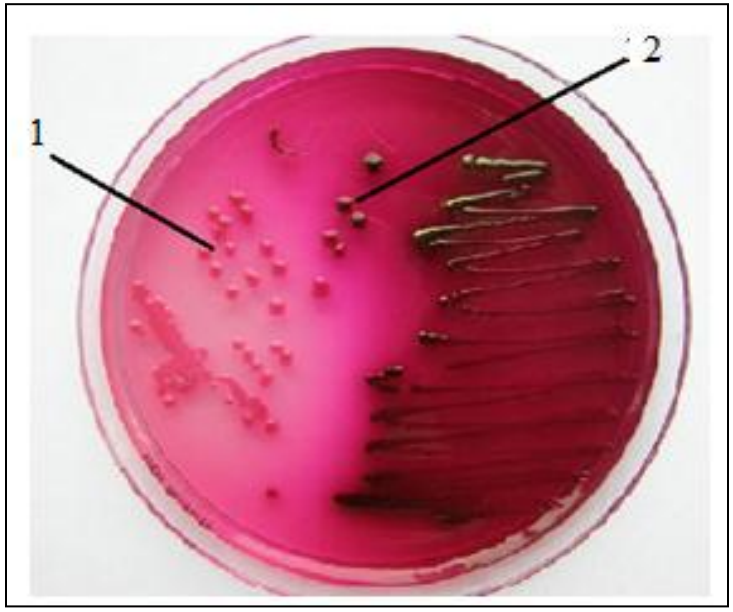
Test *Aeromonas, Plesiomonas, Pseudomonas, Campylobacter, Pasteurella, Neisseria is used in the initial identification of Gram-negative bacteria*

Differential media

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

Endo

•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



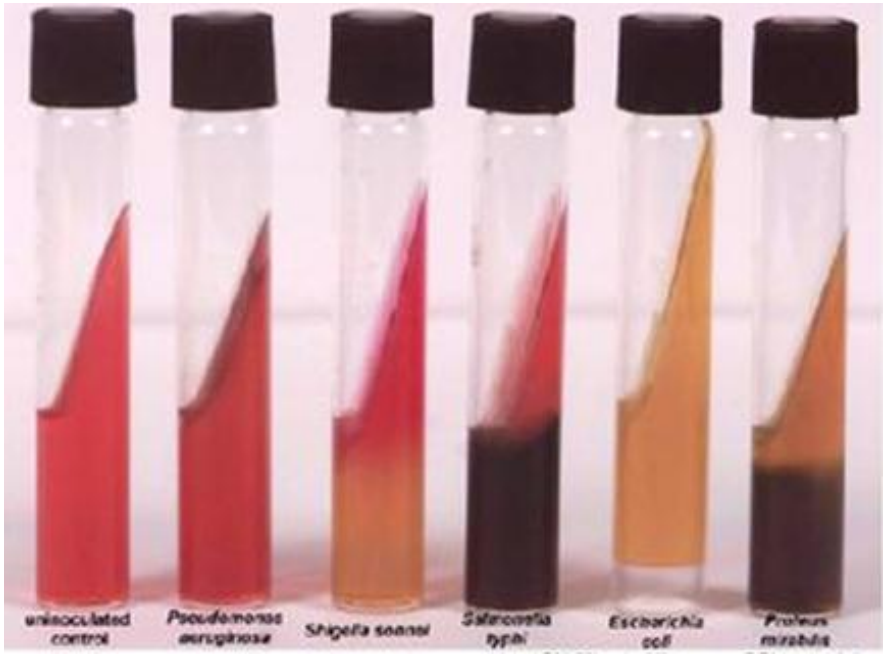
Kligler

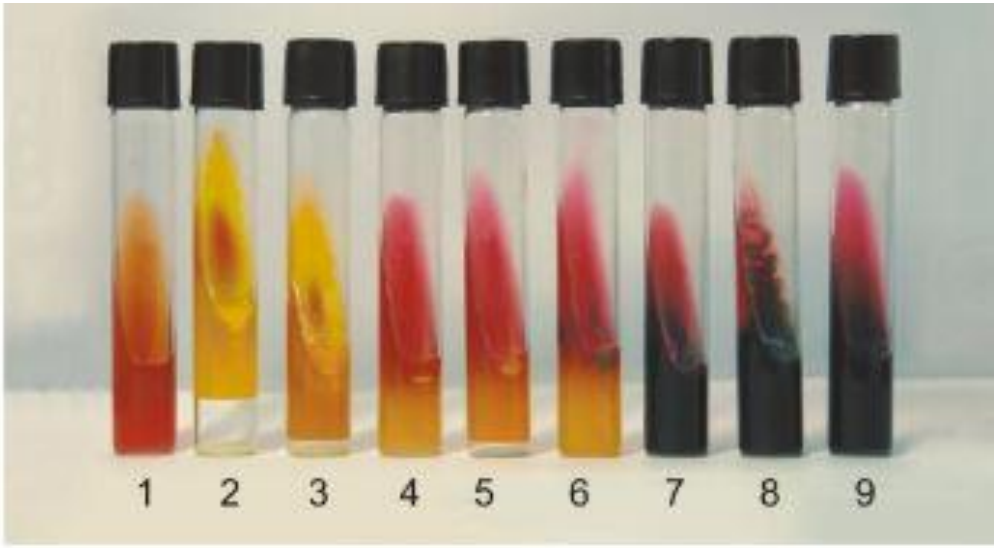
Contains: 1% lactose, 0,1% glucose, Na-tiosulfat, Fe-sulfat, İndikator

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)





Kligler Iron Agar (M078)

1. Control
2. Escherichia coli ATCC 25922
3. Enterobacter aerogenes ATCC 13048
4. Shigella flexneri ATCC 12022
5. Salmonella Paratyphi A ATCC 9150
6. Salmonella Typhi ATCC 6539
7. Proteus vulgaris ATCC 6380
8. Citrobacter freundii ATCC 8090
9. Salmonella Enteritidis ATCC 13076

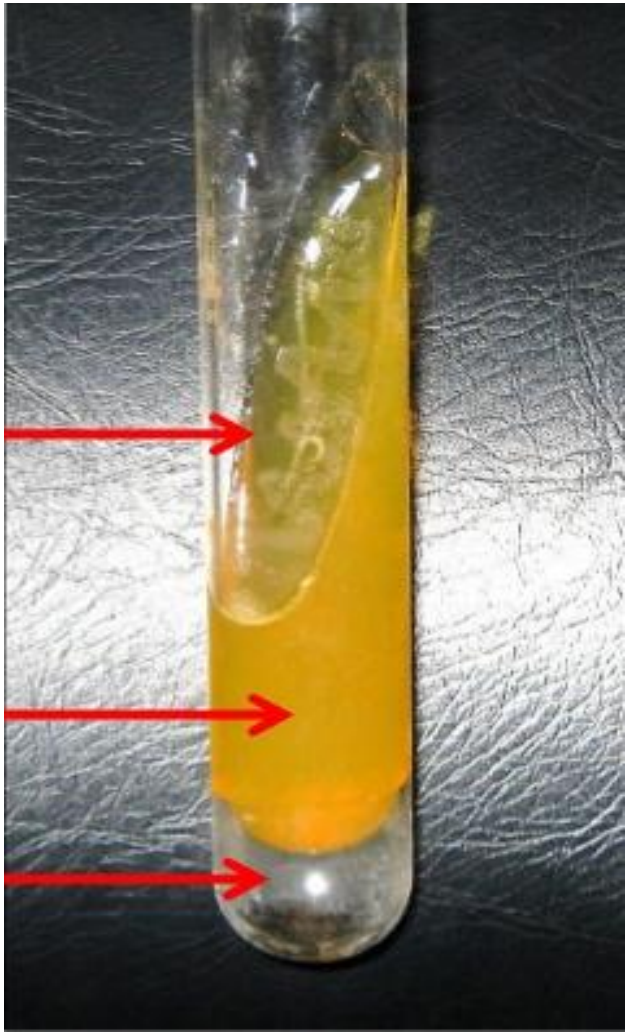
Kligler agar

Escherichiae coli

SLANT: ACID (+)

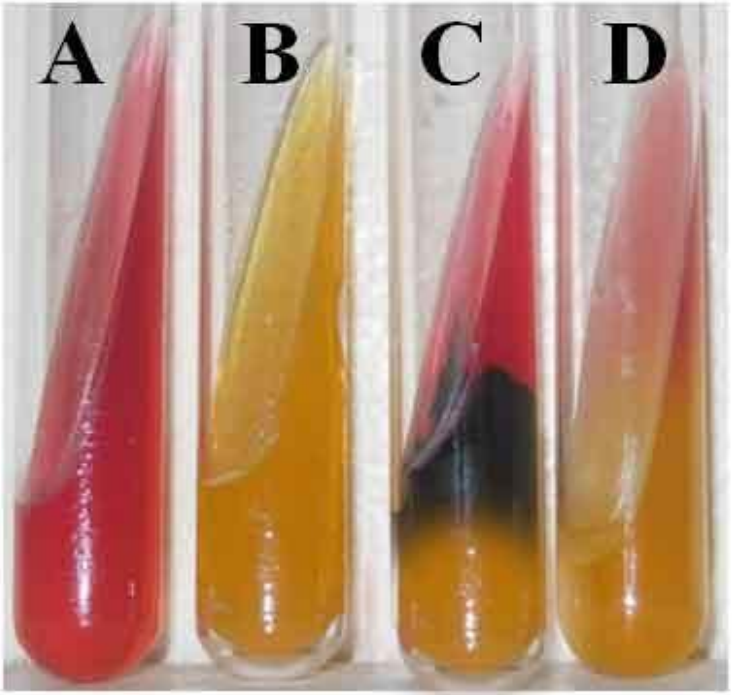
COLUMN: ACID (+)

GAS (+)



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 H_2S (black color)
- pH-indicator phenol red



A) ***Psuedomonas aeroginosa*****:** Gluc (-), Lac/Suc (-), H_2S (-)

B) ***Escherichia coli*****:** Gluc (+), Lac/Suc (+), H_2S (-)

C) ***Salmonella typhimurium*****:** Gluc (+), Lac/Suc (-), H_2S (+)

D) ***Shigella boydii*****:** Gluc (+), Lac/Suc (-), H_2S (-)

TSI – Triple Sugar Iron Agar –triple sugar (glucose, lactose, sucrose) iron agar

TSI agar – before inoculation

SLANT:
Lactose/Sucrose does not break down (-)

Hidrogen-sulfid (-)

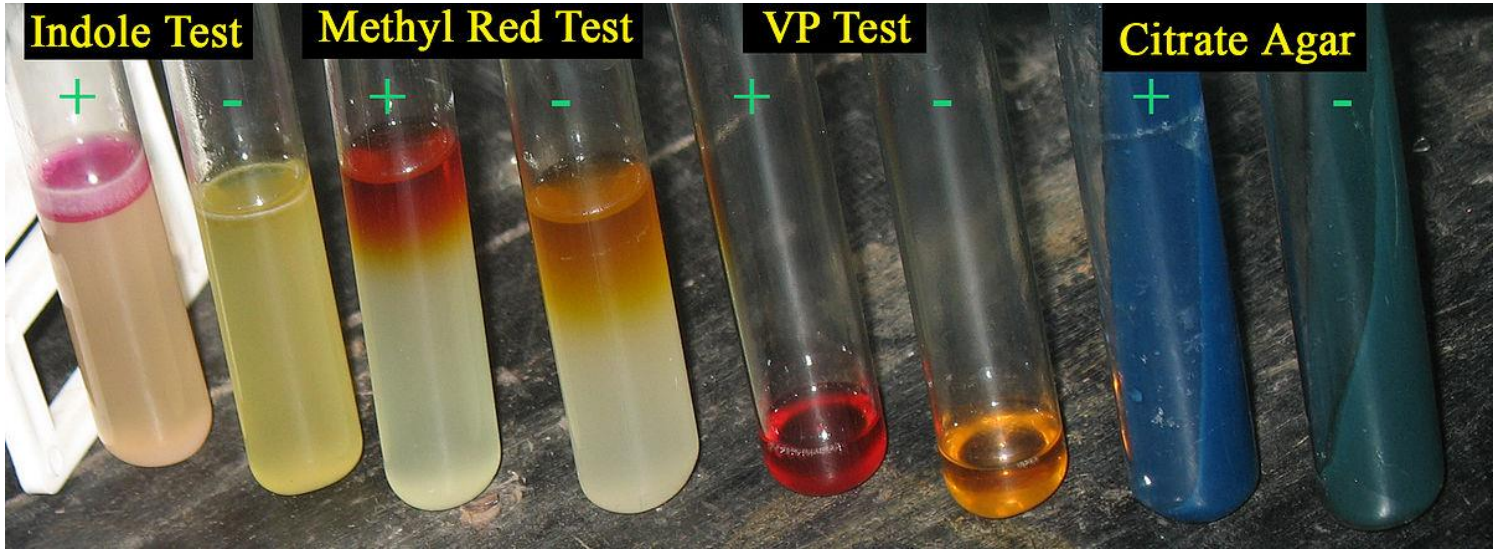
Gas (-)

COLUMN:
Glucose is broken down to acid (+)

TSI agar – after inoculation

İMVİC test(4 test)

- İndole
- Methyl red
- Voges-Proskauer
- Citrate

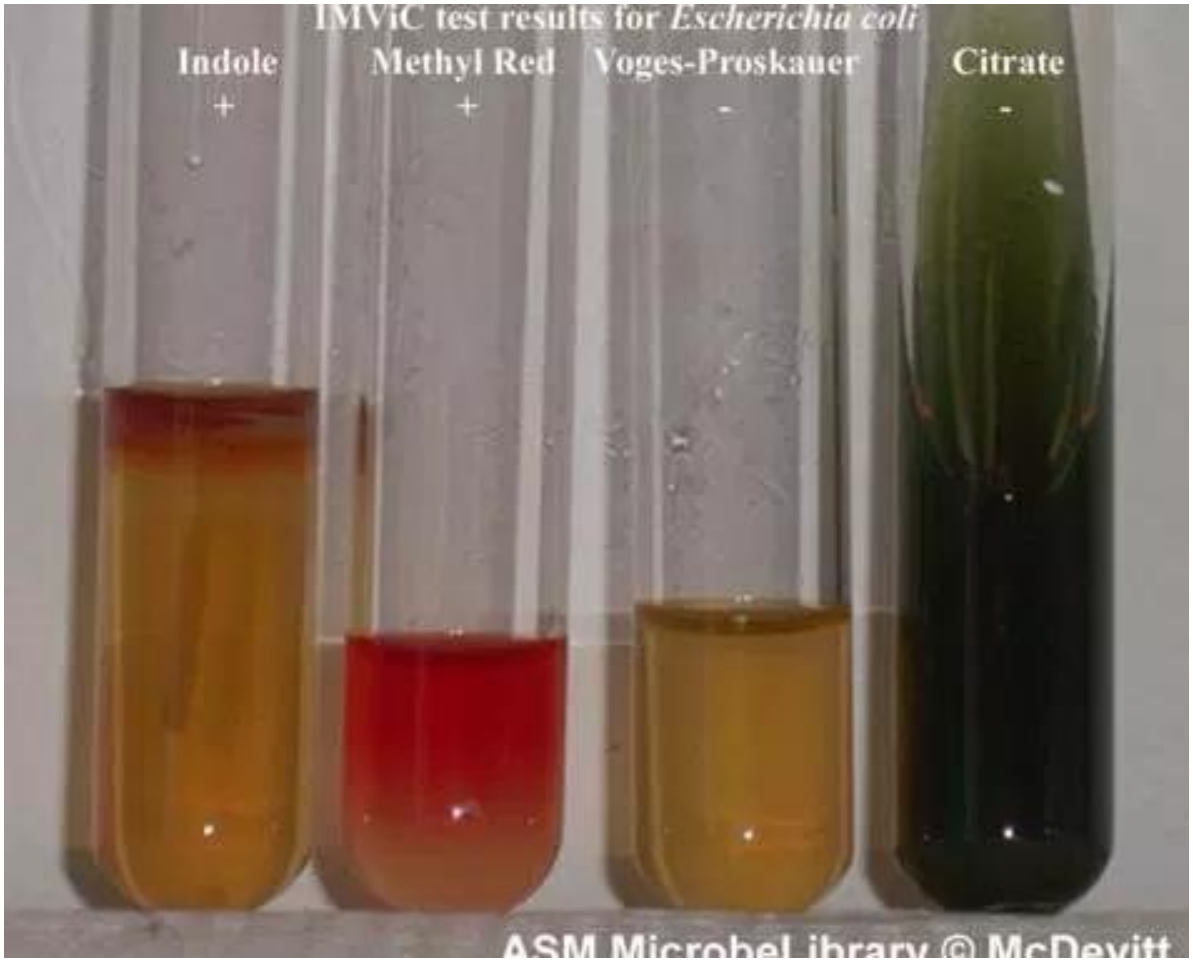


IMVIC results of different bacteria

IMViC Reactions

	I	M	Vi	C
Escherichia coli	+	+	–	–
Edwardsiella tarda	+	+	–	–
Proteus vulgaris	+	+	–	–
Klebsiella pneumoniae	–	–	+	+
Klebsiella oxytoca	+	–	+	+
Enterobacter spp.	–	–	+	+
Serratia marcescens	–	–	+	+
Citrobacter freundii	–	+	–	+
Citrobacter koseri	+	+	–	+

E.Coli İMVİC test identification



API sistem

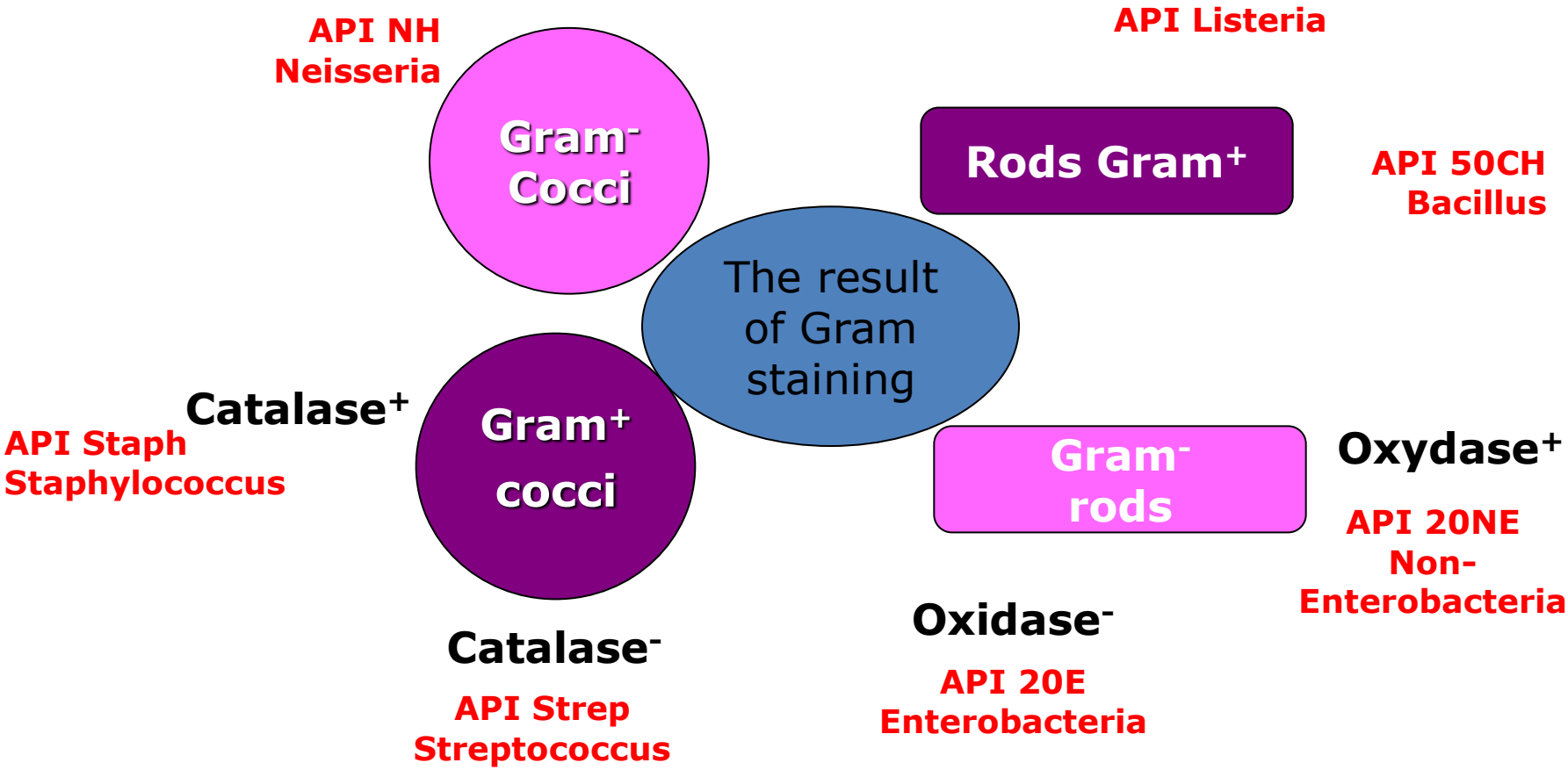
(Application programming interface)

… 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

API system

Different APY panels are used depending on results of Gram staining



API system

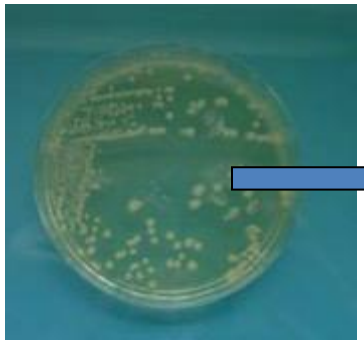
API Strep
→ *Streptococcus* spp. identification

API Staph
→ *Staphylococcus* spp. identification

API 20NE
→ non-Enterobacterales spp. identification (*Pseudomonas*)

API 20E
→ Enterobacteria spp. identification

API system identification stages



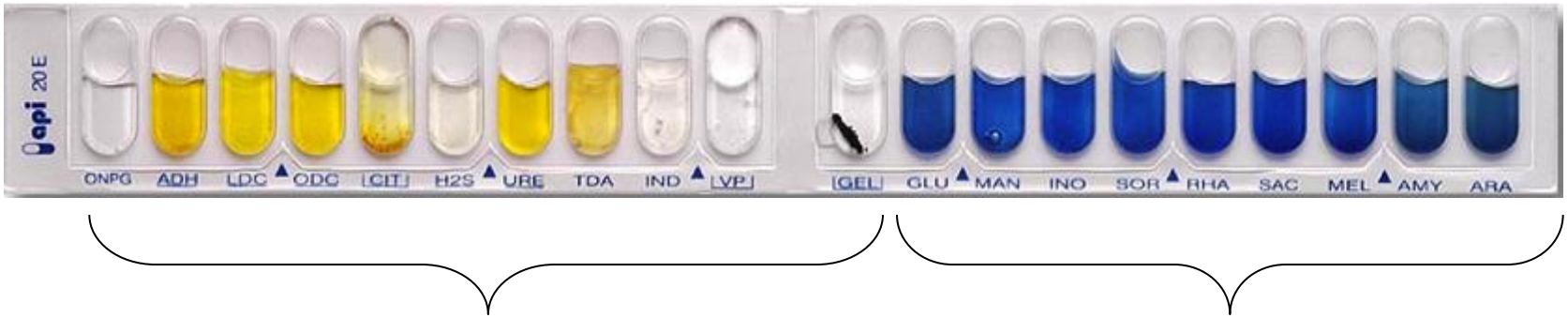
1 bacateria colony

5 ml sterile water

Inoculation of bacterial suspension in microtubes

APİsystem

API system after 24 hour incubation at 37 degree



Nitrogen metabolism

Carbohydrate metabolism

24h / 37˚ C.....

APİ system

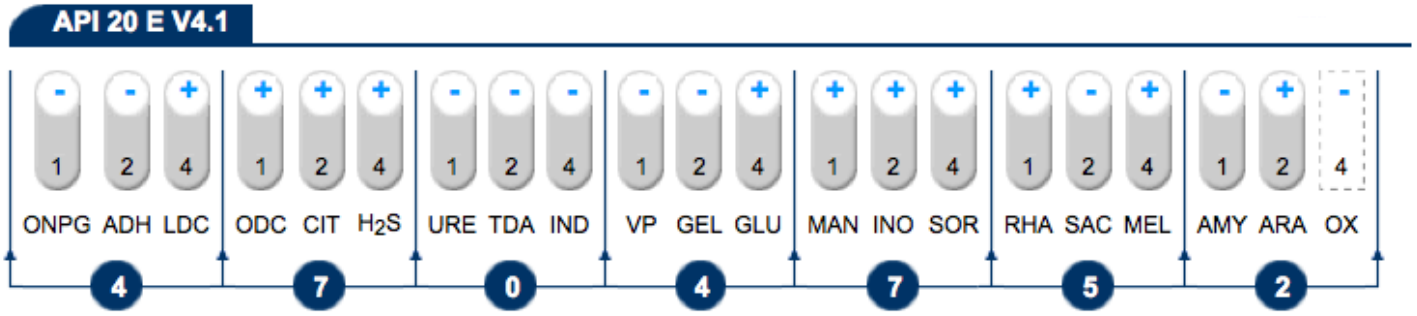
API 20 E after incubation…All test are positive:

after incubation …All tests negative

API system

(results interpretation)

Entering of results in online database:



APİ system

(results interpretation)

Expression of results by software :

EXCELLENTE IDENTIFICATION	
Galerie	API 20 E V4.1
Profil	4 7 0 4 7 5 2
Note(s)	CONFIRMER PAR DES TESTS SEROLOGIQUES

Taxons significatif(s)	% ID	T	Test(s) à l'encontre			
Salmonella spp	99.9	0.95				

Identified bacteria

Identification quality

APi system

(results are interpreted using online database 0

Reading an API20E using the online database (1).mp4

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.

Novel automated system for microorganism identification

- 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.

BioMerieux (USA) *Vitek-2 Compact* automated microbiological analyzer

Matrix activated laser desorbtion/ionization (MALDİ-TOF)

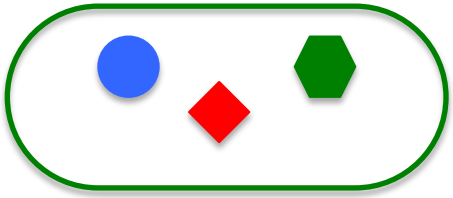
Automate system using mass spectrometry

Principle

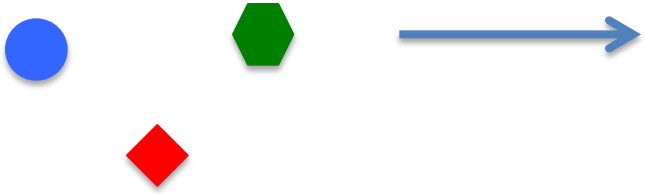
Physical detection of cell proteins via mass spectrometry
+ obtained spectrum is compared with database

MALDİ-TOF

Bacteria

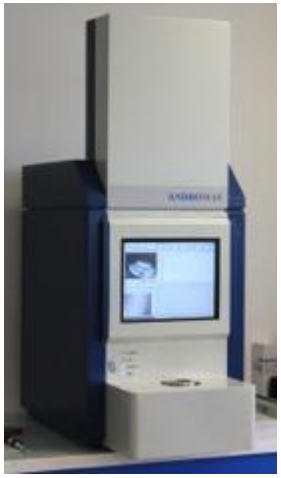


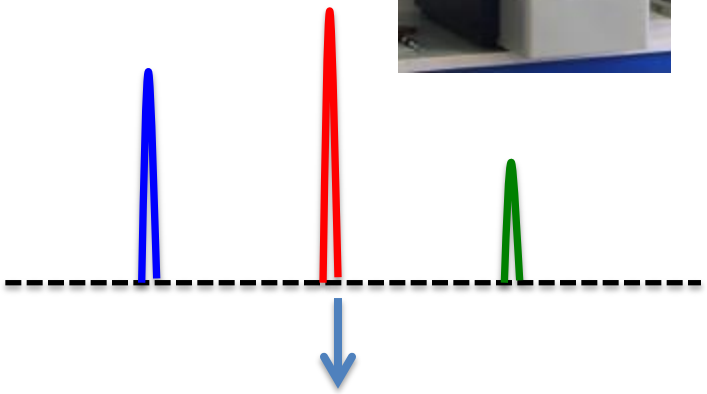
Cell lysis



Molecule extraction

Detection of molecules by Mass-spectrometry and construction of spectres

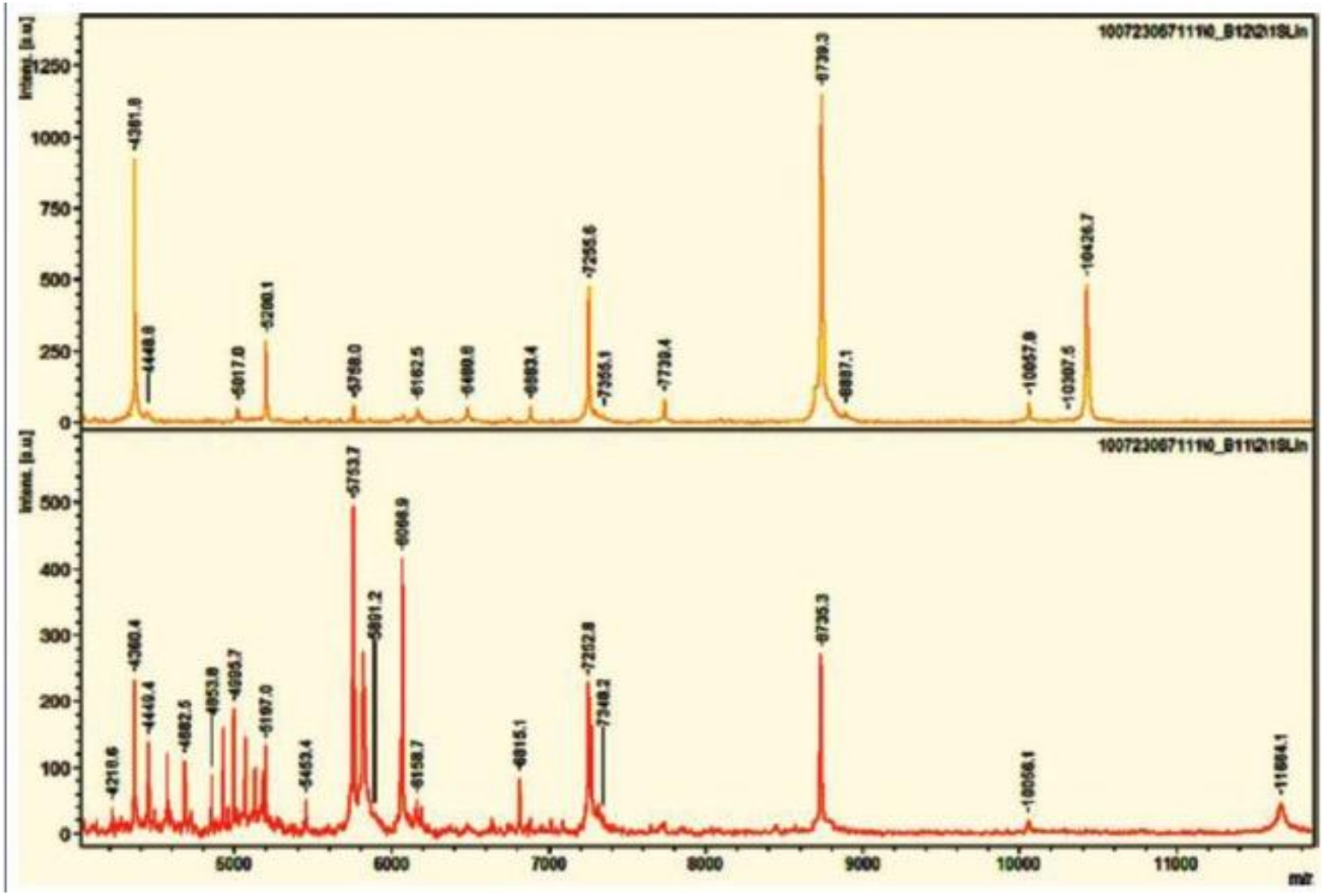




Alignment of resulted spectrum with database and identification

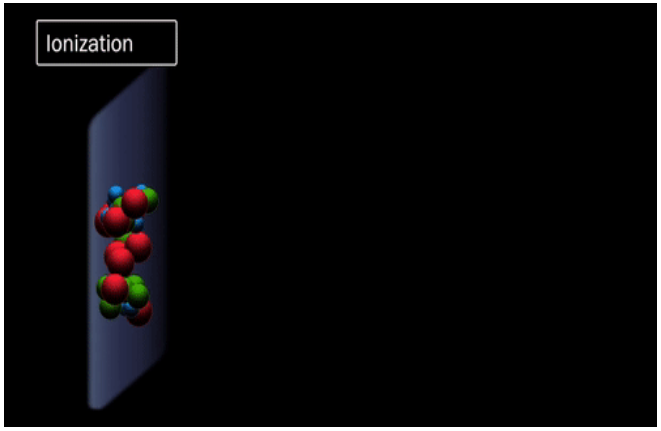
MALDİ –TOF

(Bacteria spectrum)



MALDI-tof mass spectrometry

- 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.

Biomerieux VITEK-2 - bacteriological analyzer

Antimicrobial susceptibility test – (strips) antimicrobial susceptibility testing-AST test.

Species identification (ID) cards