Practical part of Virology and Bacterial metabolism

First part: Virology

Contents

Virology

Viruses:

They are entities' its genome is DNA or RNA single or double strand enveloped by a protein coat called capsid consists of structural units called capsomers.

Virus's classification:

The highest level of viral classification recognizes six major groups according to the nature of the genome:

1. Double stranded DNA viruses

   There are no plant viruses in this group. This group is defined to include only viruses that replicate without RNA intermediate, it is include those viruses with the largest known genomes about 400,000 base pairs and there is only one genome components, which may be linear or circular, well known viruses in this group include herpes and pox viruses.

2. Single strand DNA viruses:-

   There are two families of plant viruses in this group and both of these small circular genome components often with two or more segments.

3. Reverse-transcribing viruses:-

   These have dsDNA or ssRNA genomes and their replication includes the synthesis of DNA from RNA by the enzyme reverse transcriptase; many integrate into their host genomes. There is a single family of plant viruses in this group and this is

characterized by a single component of circular dsDNA, the replication of which is *via* an RNA intermediate.

4. Double-stranded RNA (dsRNA):-

   Some plant viruses and many of the mycoviruses are included in this group.

5. Negative sense single-stranded RNA (- ssRNA):-

   In this group, some or all of the genes are translated into protein from an RNA strand complementary to that of the genome (as packaged in the virus particle). There are some plant viruses in this group.

6. Positive sense single-stranded RNA (+ssRNA):

   The majority of plant viruses are included in this group.

   Within each of these groups, many different characteristics are used to classify the viruses into families, genera and species. Typically, a combination of characters is used and some of the most important are:-

Within each of these groups, many different characteristics are used to classify the viruses into families, genera and species. Typically, a combination of characters is used and some of the most important are:-

- Particle morphology: the shape and size of particles as seen under the electron microscope.
- Genome properties: this includes the number of genome components and the translation strategy. Where genome sequences have been determined, the relatedness of different sequences is often an important factor in discriminating between species.

- Biological properties: this may include the type of host and also the mode of transmission.
- Serological properties: the relatedness (or otherwise) of the virion protein(s).

Isolation and purification of plant viruses:

Isolation or purification of plant viruses is necessary to know about their structure and properties. By employing the method of purification ,a virus is finally obtained in its pure form as a colourless pellet in a test tube and may be used for various purposes following are steps involved in virus purification (isolation) :-

1. Infected leaves are homogenized in water or in phosphate, borate or citrate buffer in an electric grinder or in a motor with pestle.

2. Tissue homogenate is strained through a piece of muslin cloth or cheese cloth. Crude sap which comes out and contains virus is collected and then poured into centrifuge tube. The tube is spun at low –speed (3000-170000g). As a result, the crude sap differentiates into supernatant and a pellet. The pellet is discarded and the supernatant with virus is collected.

3. The supernatant with virus is poured into centrifuge tube .The tube is placed in fixed angle rotor of ultracentrifuge and spun at high speed (40000-150000) after the tube settles, the virus sediments and forms tiny pellet at the bottom of the tube and a supernatant over it. Supernatant is discarded and the pellet of virus is mixed with buffer and stirred with rod so that it resuspends in buffer.

4. Low and high speed centrifugation steps are repeated 2-3 times and the virus is purified by density gradient centrifugation, the most frequently used technique. A tier of layer of sucrose solutions of different concentrations (e.g., 10-40), and hence densities, is formed in the centrifuge tube ,layer at the bottom being the most dense and one at the top of the least dense with layer of intermediate concentration .Virus suspensions is placed at top of the top most layer and centrifuged in swimming –bucket rotors at high speed ultracentrifuge .
5. When settled, virus partials move together as a band in gradient solution of sucrose. The virus band is collected as separate fraction through puncture at the bottom of the centrifuge tube .The virus fraction is placed in cellulose dialysis tubing and sucrose is removed by dialysis in buffer solution or water . Thus the virus is obtained in pure form.

External symptoms of plant viral diseases:

1- Chlolosis:-

These symptoms represent the area which becomes weakened in green colour due to either destruction or inhibition of chlorophyll formation subsequent to infection and the cells with chlorotic symptoms contain less chlorophyll thus appearing pale green or yellowish in colour.

2- Mosaic:-

These symptoms represent irregular intermingling of light green, yellow or white areas with the normal green colour of the plants or fruits forming a mosaic like pattern.

3- Mottling:-

It is an irregular pattern of indistinct light and dark areas. Mottling is considered equivalent to the mosaic by some virologists.

4- Vein chlorosis and vein clearing:-

The former represents the chlorosis restricted to the veins while the latter represents the translucence of the veins rather than being chlorotic or yellow. These two symptoms make the veins appear lighter in colour against dark background of normal green tissue.

5- Vein mosaic:-

These represent the chlorosis along the main vein with the lighter areas irregular in shape and distribution. Sometimes vein mosaic extends to adjacent areas in the form of irregular patterns.

6- Vein banding:-

In these symptoms the chlorotic area occur along the veins in a regular manner resulting in the presence of chlorotic and regular green coloured bands.

7- Necrosis:-

These are the common viral symptoms occurring in plants representing the death of the cells in localized areas .These areas of dead tissues are generally differentiated by the presence of dark brown border around them . Necrosis may remain localized but sometimes it spreads as long streaks producing systemic necrosis resulting in the death of the whole plant. One strain of TMV develops streaks on the stems and causes complete necrosis of leaves and fruits resulting in the death of the infected tomato plants.

8- Ring spots:-

These symptoms represent the localized circular spots formed by concentric rings of chlorotic and normal green tissue .ring spots are generally accompanied with necrosis. They may be presents singly or in groups developing concentrically on infected parts.

9- Enations:-

Enations are the out growths generally occurring on vein or midrib on the lower surface of the leaves.they may vary in number and shape .they may be small, large, papillate or spin like in shape .in some cases like pea enation mosaic virus the outgrowths develop between or adjacent to the veins on the lower surface of the leaves and look like leaves , funnels,wings,cups etc.

10- Leaf narrowing:-

In these symptoms the infected leaves generally become narrow due to reduced growth of laminar tissue. The veins and the midrib remain normal in growth, in the case where leaf narrowing is at extreme as represented by tmv infected tomato only the midriband veins are present and the laminar tissue is almost absent.

11- Leaf curling :-

These symptoms represent irregular and extensive wrinkling and furrowing of leaves due to reduced growth of veins in comparison to the growth of laminar tissue resulting in shrunken veins and raised up laminar tissue leading to the curling of the leaves.

Example: leaf curl of papaya, leaf of tomato etc.

12- Leaf rolling:-

In these symptoms the downward and upward rolling of leaves takes place and this convers their entire length example leaf roll of potato.

13- Stunting or dwarfing:-

In this case the infected plants show general retardation in all this organs resulting in stunting of plants. The morphology of the stunted plants remains normal. Example: chrysanthemum stunt virus.

14- Rosetting:-

Shortening of internodes due to reduced growth brings the leaves together at the tip of the branches giving rosette like appearance.

15- Tumors:-

These are large and irregular outgrowths developed due to abnormal increase in size and number of the cells .tumors generally occur on roots of some leguminous plants.tumors develop on roots of remix sp.infected with wound tumor virus.

16- Pollen abortion and pollen sterility:-

In some infected plants either the pollen may not be produced (tomato ring spot)or may remain sterile (quirking virus on datura sp.)

17- Premature abscission of leaves:-

This is acommon occurrence in many plant viral diseases.

18- Colour deviation:

* Chlorosis:

Appearance of light areas on the plant surface due to lack of plastid .chlorosis has two shapes either spotting (regular light areas) or motting (irregular light areas).

* Mosaic:

Areas with different colours ,larger than chlorosis colours ranged from white to deep green.

* Yellowish:

All the leaf becomes yellow due to destruction of plastid.

* Colour breaking: changes of flower colour.

19- Death of tissue:

*Necrosis: destroying of group of cells or tissues or whole of plant body.

*Local lesions: Death of certain tissues or cells.

*Streaks:

The dead part of the leaves appears as longitudinal lines ranged form (7-10) lines with brown colour.

20- Deformations or malformations:

*Leaf crul or rolling of leaves.

*Fly form shape of leaf blade.

*Enation: abnormal growth on the leaf blade.

*Blister: pits formed due to group of cells the pits are more green spots than other parts of leaf blade.

21- Vein clearing:

The region around the vein appears colourless or white.

Internal symptoms of plant viral diseases:

Instead of the external symptoms described earlier there are many symptoms developed endogenously with the virus infected plants they are called internal symptoms. Some important ones are being given in brief:

1- **Change in parenchymatous cell:**

Mainly mosaic viruses alter the structural and functional characteristics of various parenchymatous cells e.g.

a) Palisade parenchyma formation is poor. They also look like spongy parenchyma i.e. spherical
b) Cells of chlorotic areas remain smaller in size.
c) Intercellular spaces are reduced in size.
d) Hyperplasia may result in the infected cells.
e) Chlorophyll distruction may occur affecting the development of chloroplast and thus bringing various abnormalities in them.

2- **Changes in xylem:**

Viruses promote the formation of styluses and gummosis, tyloses the bladder like outgrowth. Block the xylem lumen. Gummosis represents the formation of gum like substances as a result of

carbohydrate decomposition. These gums like substance sandty loses block the lumen of the xylem vessels stopping ascent of water and other inorganic substances. This results in finally wilting and even death of the host.

3- Change in phloem:

Necrosis is the ultimate fate of those phloem cells which are infected with viruses. These infections are however localized and mainly occur in the vicinity of sieve elements. Sometimes hyperplasia has been observed before necrosis in infected phloem elements. Since phloem elements represent the food channel of the plant, any disturbance in it affects the food supply of the host or formation of callose in the sieve tube and companion cells.

4- Changes in cell and cell organelles

Following are some important changes that have been observed either in the cell organelles due to viral infections:

(a) Infected cell increase vacuole formation in their cytoplasm.

(b) The concentration of the cytoplasmic matrix becomes low.

(c) Ribosomes are reduced in number.

(d) There occur change in shape and size of nuclei of the infected cells.

(e) Abnormality may operate in number and morphology of mitochondria of infected cells. Mitochondria is the unit of energy deceasing the no. of it lead to decrease energy of plant.

(f) Meiosis and mitosis may also be affected in viral infected cells instead of above described internal symptoms, many amorphous and crystalline inclusions such as x-bodies, spindle or needle like crystals etc, have been reported within the cells infected by viruses. Such

inclusions have never been observed in cells which are free from the concerned viral infection. On the lower surface of the leaves.

(g) Formation of vesicles of vacuoles inside plastid.

5- Destroying of cell membrane which is permeable membrane.

Examples for plant viral diseases:

Potato virus y (PVY)

Pvy is a member of the *Potyviridae* family of viruses.

Common symptoms:

Once infected, a potato plant can express symptoms in as few as 10 days, depending on the variety. Symptoms of PVY infection are variable and range from mild (foliar mottling, streaking and mosaic) to sever (leaf necrosis, leaf drop and stunting). The severity of the symptoms depends on the potato cultivar, environmental conditions and the strain of PVY infecting the plant.

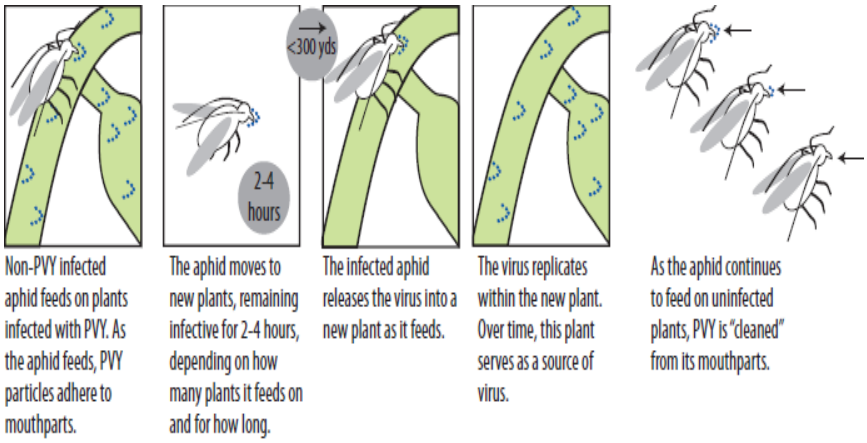
An example of how different PVY strains cause different levels of symptom severity. On the Pike variety, PVY^{N-WI} symptoms (top) are mild. PVY^{O} symptoms are more severe (bottom).

Figure 3. A Yukon Gold tuber (top) when infected with certain strains of PVY is prone to develop potato tuber necrotic ringspot disease (bottom).

Usual means of spread:

PVY is spread from plant to plant by mechanical means or by aphids. Mechanical transmission generally occurs when an infected plant and an adjacent healthy plant are wounded by wind or human activity. The wounds of an infected plant leak sap that contains the

virus and the wound of a nearby healthy plant may take in some of that virus when the two plants touch. The more efficient and rapid form of transmission in fields involves aphid vectors. When an aphid feeds on a Pvy infected plant, virus particles adhere to the tips of its mouthparts. If the aphid then moves to a healthy plant and begins to feed, the virus particles are released and transferred to the healthy plant, leading to Pvy infection. After an aphid acquired the virus, it generally transmits it to uninfected plants for only a short period of time, usually less than two to four hours.



Principal hosts:
Solanaceous crops including tomato, pepper, tobacco and potato.

Control:

Excluding sources of the virus from the planted crop (by using seed certified to be free of Pvy is one strategy to exclude the virus from the farm), reducing the attractiveness of the growing crop to migrating aphids and reducing the likelihood that infectious aphids will feeed on the crop.

Using seed certified to be free of PVY is one strategy to exclude the virus from the farm.

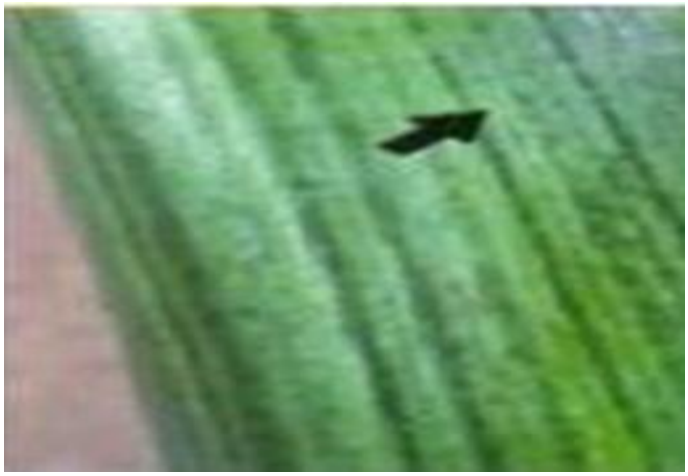
Banana bunchy top disease (rosetting)

What is banana bunchy top disease?

- The responsible for this disease is banana bunchy top virus.
- It is one of the most important and dangerous virus that infect banana.
- The virus becomes widely spread in presence of aphids.

Common symptoms:

- The initial symptoms are appearance of dark green lines on the down surface of midrib.



- The edges of the leaves become yellow then brown and become easy to break.
- As progress of infection the small leaves become crowded at the false tip of the brunches.
- Forming the rosette shape (rosetting).
- The leaves emerges difficulty from infected adult plants and the edges become wavy shape and narrow.
- Usually the infected plants become unproductive and if the plants product fruit the fruit will be disfigured.
- The next generation of the infected plants will be stunting and the leaves will be hard and erected and not flat and shorter than the leaves of normal plant.

Usual means of spread:

- It doesn't transmit mechanically by cellular sap.
- It transmit with banana aphid (*Pentalonia nigronervosea*).

Control:

- Plant uninfected seeds.
- Put a cup of kerosene inside the plant to avoid aphids and cut the plant from the middle.
- Take off the infected plants with roots (the full plant) and burn the location of the removed infected plants.
- Pour kerosene on the soil (the location of the removed infected plants) and leave the soil exposed to air for two weeks and put (CaO) inside the plant.

Banana bract mosaic virus

Banana bract mosaic virus (BBrMV) is also called banana bract mosaic and Kokkan disease. This disease was first found in the Philippines in 1979**.**

Description

Initial symptoms of banana bract mosaic virus include green or red-brown streaks or spindle shaped lesions on the leaf stalks and sometimes on the midribs of the new banana leaves. Chlorotic (pale yellow or yellow-white) streaks may also appear on the bunch stems and fingers. In severe cases the affected fruit can be rejected. If the outer leaves are removed from the banana plant stem; red-brown, spindle-shaped streaks can be seen on the exposed stem.

Life cycle

Banana bract mosaic virus infects the vegetative, flowering and fruiting stages of banana plants. The disease is transmitted by aphids which acquire the virus while feeding on infected plants. The virus only lives inside the aphids for a short period of time and is transmitted in a nonpersistent manner. Banana bract mosaic virus can be spread by four aphids that are present in Australia: banana aphid (*Pentalonia nigronervosa*), corn aphid (*Rhopaloshiphum maidis*) cotton or melon aphid (*Aphis gossypii*), cowpea aphid (*Aphis craccivora*). Double infections of banana bract mosaic virus and banana bunchy top virus (also transmitted by the banana aphid) can occur in banana plants.

Hosts

Banana bract mosaic virus infects cultivated and wild bananas (Musaceae plant family).

Spread

Banana bract mosaic virus is spread longer distances by infected banana planting material, including suckers, bits, corms and unindexed tissue culture plantlets.

Distribution

Banana bract mosaic virus is found in India, the Philippines, Sri Lanka, Thailand, Vietnam and Western Samoa.

Actions to minimise risks

Put in place biosecurity best practice actions to prevent entry, establishment and spread of pests and diseases: practise “Come clean, Go clean” ensure all staff and visitors are instructed in and adhere to your business management hygiene requirements source propagation material of a known high health status from reputable suppliers monitor your crop regularly keep records isolate banana plants or areas with suspect symptoms to prevent further spread

Figure 1 A dark, red-brown mosaic pattern on the banana flower bract is the main symptom of banana bract mosaic virus

Figure 2 Red-brown, spindle-shaped streaks on the exposed banana stem (when the outer leaves are removed) indicating banana bract mosaic virus

Tomato spotted wilt virus (TSWV):

Taxonomic position: Viruses: Bunyaviridae: *Tospovirus*

Symptoms

On tomatoes, plants show bronzing, curling, necrotic streaks and spots on the leaves. Dark-brown streaks also appear on leaf petioles, stems and growing tips. The plants are small and stunted as compared with healthy plants. The ripe fruit shows paler red or yellow areas on the skin. Sometimes, affected plants are killed by severe necrosis.

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Usual means of spread:

- TSWV is spread by tiny insects called thrips, which pick up the virus by feeding on infected plants.
- If the virus and the thrips are present, the severity of the disease depends on the weather.

- TSWV is not seed-transmitted.

Principal host:

TSWV is polyphagous on a great number of mostly herbaceous hosts. *Capsicum annuum*, lettuces, tobacco, tomatoes and various ornamental crops are the main hosts.

Control

ž The presence of thrips in the crops should be monitored using yellow sticky cards. If the disease appears in a crop, infected plants should be rogued and destroyed immediately and the house treated with insecticide against thrips.

Sugarcane mosaic virus

List of symptoms/signs

- Leaves: abnormal colours, abnormal forms, abnormal patterns and necrotic areas
- Stems: discoloration of bark and stunting or rosetting
- Whole plant: dwarfing

The particular symptoms depend on the virus strain, the host cultivar and the environmental conditions, particularly temperature.

Prevention and control

Control of SCMV:

- Applying herbicides may be useful in maintaining mosaic-free seed plots of cane if the level of infection is lower than 5%.
- Use of mosaic-free seed cane is an effective control measure where inoculum pressures are not intense. Thermotherapy of planting material can result in some plants that are free of SCMV.

Control of Aphid Vectors

- Altering the times of planting and harvesting so that they do not coincide with high aphid vector populations and can reduce losses.
- Should not be grown near infected sugarcane crops.
- The use of insecticides failed to prevent aphid vectors from spreading SCMV.
- Aphids which transmit SCMV come from outside as well as inside the sugarcane crop, care should be given to reduce the build up of the vector species in the vicinity.

Cucumber mosaic virus {CMV }

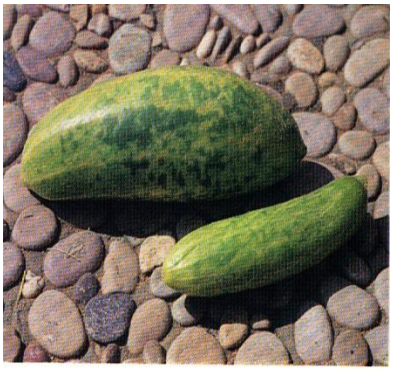
- Cucumber mosaic virus (CMV) is one of the most common plant viruses and causes a wide range of symptoms, especially yellow mottling, distortion and stunting. Expect damage whenever susceptible plants are growing well in spring and summer.

Principle Hosts

Apart from cucumbers and other cucurbits, it also attacks spinach ، lettuce and celery and many flowers, especially lilies.

Usually means of spread

- CMV is vectored by several aphid species which feed on a broad range of plants and this contributes to the very wide host range of this virus
- CMV is occasionally transmitted through seed in around 20 of plant species
- It can spread mecanically by a sap.
- It can also be transmitted in seeds and by the parasitic weeds, Cuscuta sp.
- It naturally spread in soil by zoospores of fungus.
- CMV is easily transmitted on garden tools and gardeners' fingers.



Cucumber: mosaic by cucumber mosaic virus (fruit symptoms) (Middle East)

Control

- **Chemical control: There are no chemical controls available to control virues. The use of insecticides to reduce aphid transmission is not practical.**
- **Non chemical: Avoid handling healthy plants after working with suspected infected ones until tools or hands have been washed with soapy water. Destroy suspect plants promptly to reduce the risk of transmission. Keep the garden weed free.**

Part (2): Bacterial metabolism

Introduction

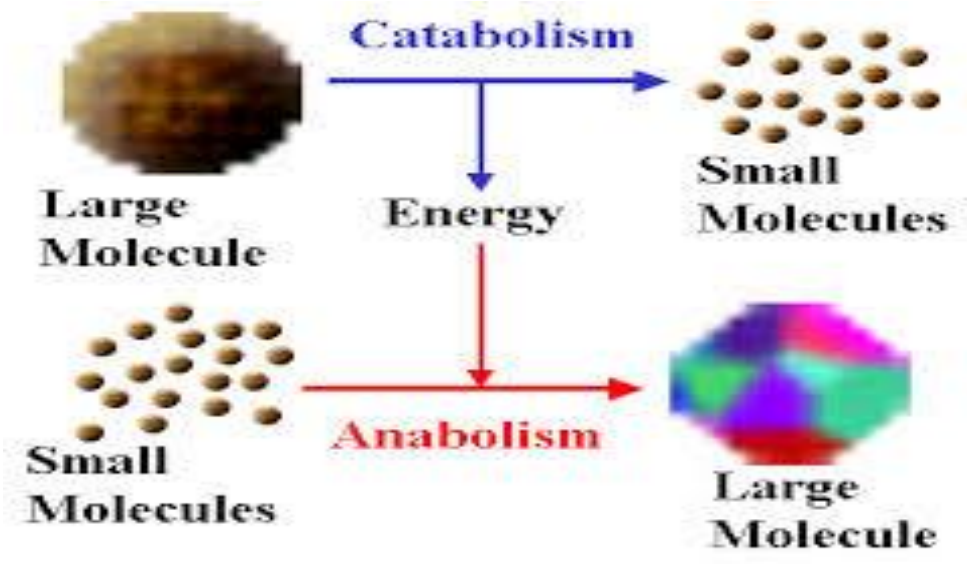
What is bacterial metabolism?

All biochemical reaction that take place inside bacterial cell by using enzymes.

- Metabolism divided into two process:

1) **Catabolism process:** breaking down of macro molecules (complex compound) into micro molecules (simple compound) to get energy and nutrient

2) **Anabolism**: assembling of micro molecules to macro molecules to get cellular structure.



- Enzymes:

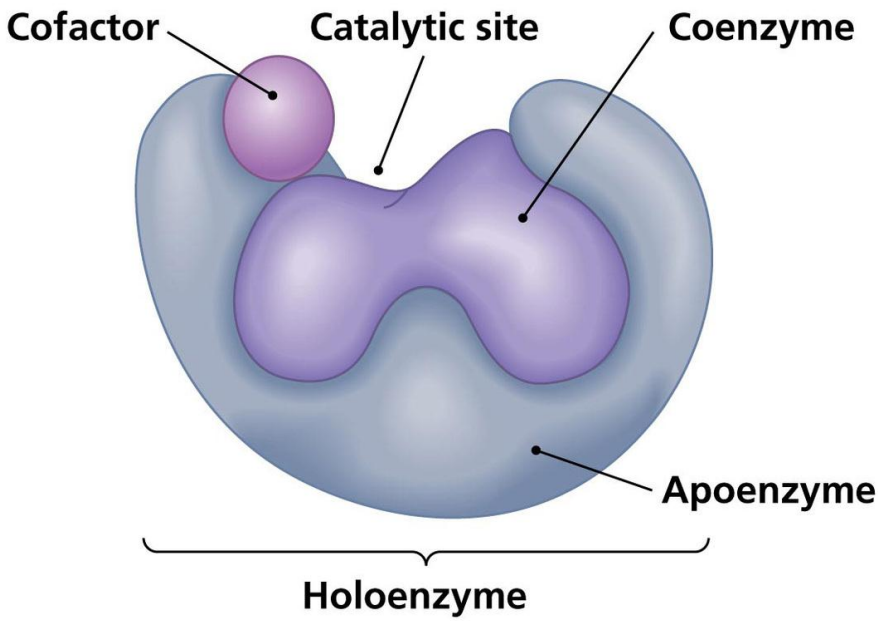
Protein compound that have active sites and catalyze the biological reaction

Structure of enzyme:

1) Apo-enzyme: The body of enzyme (protein)

2)Co-factors: Metal ion (Fe, Cu,…) or Organic material (FAD, NAD)

3) Active sites: contain saccharides



Factors effect on enzyme activity

1. Temperature
2. pH
3. Concentration of substrate
4. Concentration of enzyme
5. Inhibitors

Inhibitors

They are substances that bind to enzyme to decrease its activity

Types of inhibitors:

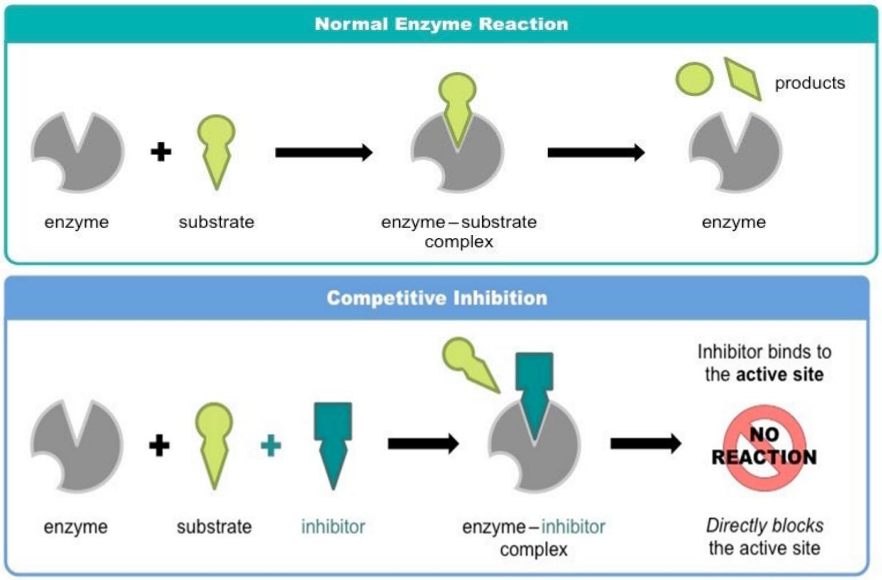
1) **Specific inhibitors (reversible): include**

- Competitive inhibitor
- Non competitive inhibitor

2) **Non specific inhibitors (irreversible)**

Competitive inhibitors

Inhibitor and substrate have the same shape so substrate and inhibitor compete for access to enzyme active site.

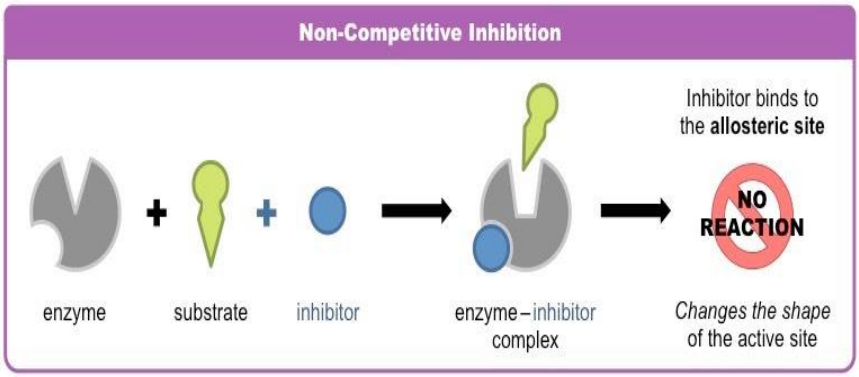


Noncompetitive inhibitors

Inhibitor binding to a site other than the active site (an allosteric site) and form ES complex

this lead to change shape of substrate binding site

So chemical reaction doesn't occur

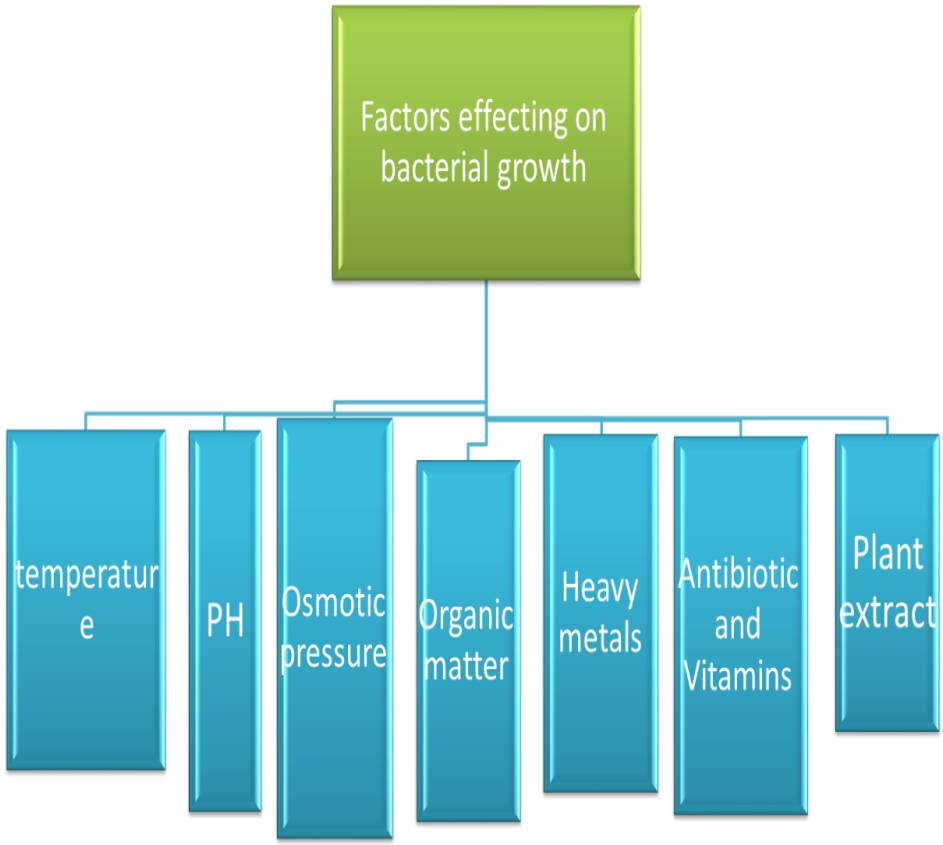

Non-Competitive Inhibition
Inhibitor binds to the allosteric site
NO REACTION
enzyme
substrate
inhibitor
enzyme–inhibitor complex
Changes the shape of the active site

Determination of bacterial protein

Why determination of bacterial protein?

1-To study the effect of different factor on bacterial growth.

2-To study the probability of bacterial growth (inhibition or stimulation)



1-Temperature

Any organism has 3 range of temperature.

A-Minimum temperature: Less than minimum temperature lead to stopping bacterial

metabolism

B-Maximum temperature: more than high

temperature lead to death bacterial cell

C-Optimum temperature: the most suitable temperature for bacterial growth

There are bacteria thermophiles and some psychrophiles but most of bacterial cell are mesophiles, they grow best at moderate temperature (20-45)

2-pH

A-Neutral (pH=7): most of bacterial cell prefers neutral medium

B-Acidic (pH<7): Few bacteria prefer it

C-alkaline (PH >7): Few bacteria prefer it

The neutral pH is the more suitable for bacterial growth but there are some bacteria grow on alkaline medium while, other grow on acidic medium.

3-Osmotic pressure

migration of water from hypotonic solution to hypertonic solution

A-Hypertonic conc.: Concentration of salt in media (outside bacterial cell) is more than inside bacterial cell So water

migrate from bacterial cell to the media leading to shrinking of bacterial cell.

B-Hypotonic conc.: Concentration of salt inside bacterial is more than concentration of salt in media so water migrate from media to the bacterial cell causing stimulation in growth.

C-Optimum conc.: Concentration of salt inside bacterial equal to concentration of salt outside bacterial cell

4-Organic matter

It is type of chemical materials that effect on bacterial growth and metabolism and controlled by enzymes that secreted by bacterial cell.

e.g. (carbon source, sulfur source) they are usually saccharides such glucose, fructose as carbon source

when two types of carbon source presence on media such as glucose and fructose bacteria can choose to consume one of them firstly then consume second this called (Dioxic growth)

5-Heavy metals

They are minor elements needed by bacteria by small

concentration like (Zn, Cu, Pb, Cd)

If the concentration of these metals increased in media, the media become toxic to bacterial cell.

6-Antibiotic

Bacteria may by resistance or sensitive to antibiotic

7-Plant extract

Organic matter which contain elements may cause stimulation or inhibition of bacterial metabolism this factor may be use as antibacterial or antifungal

Determination of bacterial protein

A. Isolation of bacteria

B. Homogenization

C. Sonication process

D. Measurement of protein by spectrophotometer

A) Isolation of bacteria

Like study effect of heavy metals

Procedures:

1) Prepare N.B media in test tube with different conc of heavy metals like (0.05,0.1,0.2,0.3,0.4,…) and prepare control tube (media without heavy metals).
2) Sterilization of test tubes on autoclave.
3) Inoculate test tube with bacterial strain.
4) Incubate test tubes media at 37˚c for 48hr.
5) Isolation of bacterial growth from culture media by centrifugation or filtration (filter unit).
6) In case of centrifugation remove supernatant and wash bacterial cell (ppt.) by saline soln. 0.9% and centrifugation.
7) Repeat centrifugation 3 times.
8) Collect saline and bacterial cell in another sterile test tube.

B) Homogenization

Using homogenizer machine that convert bacterial colonies to single (separated)bacterial cells by taking 3 ml of bacterial growth and grinding bacteria by using piston and after that put it in test tube, wash homogenizer by saline soln. to complete volume to 10 ml.

C) Sonication process

- Sonicate bacteria by using sonicator (depend on ultra-sonic waves) in ice bath for 2 min only.
- That process leading to releasing of bacterial protein.

➢ Why we use ice bath in sonicator?

as this machine produce high energy leads to degradation of bacterial cell and effect on protein of the cell.

- Why time for 2 min only?

as optimum time to break down bacterial cell is 2 min after 2 min ultrasonic begin to break down protein itself (protein content decrease)

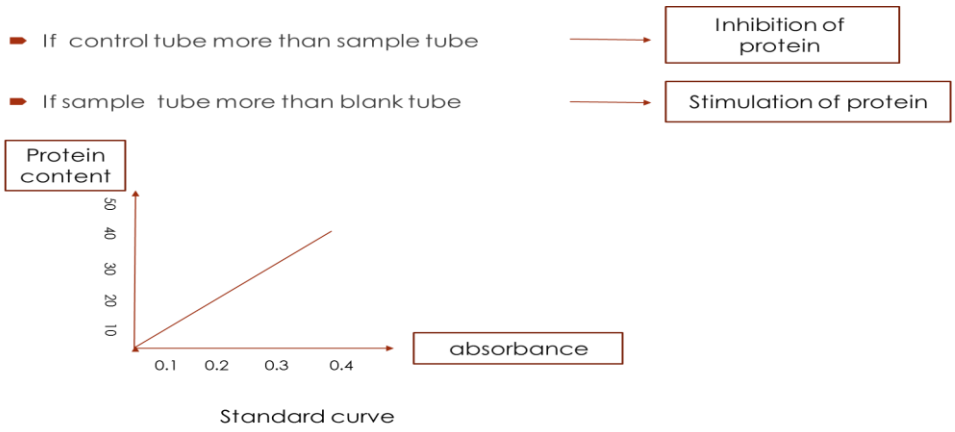
C) Measurement of protein by spectrophotometer

- Measurement depend on the difference of colour degree , if the indicator colour is blue and change to deep blue so that the change on colour consider the value of protein.
- Inside spectrophotometer there are two tubes

1-blank tube: contain indicator (coomassi blue) + 100 µl dist water

2-Sample tube:contain indicator (coomassi blue) + protein suspension

- Comassi blue absorb wave length at 595 nm.
- In spectrophotometer we put blank tube first, measure value and this called absorbance=mg/ml, micro gm/L, then make blank tube zero.
- after that we put sample tube but we should let sample tube for 5 min before put it on spectrophotometer and this time is period of reaction between protein and indicator then put tube and measure value (absorbance)= mg/ml, micro gm/L.
- Compare between control tube and sample tube.



Standard curve

Standard analysis of water

Standard analysis of water why?

To determine the contamination of water

(water may be qualified or non-qualified)

- Water is contaminated by two methods: -

1-Chemically: -like heavy metals

2-Biologically: - like microorganisms

The indicator that detect the contamination of water: -

microorganism itself such as *E. coli*, Fecal coli

Properties of these indicators: -

1- lactose fermentation and produce acid and gas

(fermentation is hydrolysis of monosaccharide in presence or absence of oxygen to organic acid, gases or alcohols

2-short rod bacteria (*E. coli*, Fecal coli)

3- Gram negative bacteria

4- non spores forming bacteria

- If four condition is available, the water will be contamination
- The experiment consists of 3 tests

1- Presumptive test

2-Confirmed test

3-Completed test

- **Presumptive test:**

Used in determination of MPN (most probable number of coli)

Steps

- 1-Prepare lactose broth media
- 10 gm lactose
- 5 gm peptone
- 3 gm beef extract
- 0.065 gm of BTB (bromo thymol blue)
- In 1 litter dist. water (Single strength)
- Or in 500 ml dist. water (Double strength)
- pH equal to 7

2-Take about 15 test tubes and named first 10 tubes single strength and last 5 tubes double strength

3-Put in 15 tubes 10 ml of media (single and double strength)

4- Sterilize media in tubes with autoclave

5-Inculate tubes by water samples by:

a- Adding 0.1 ml of water sample in first five tubes of single strength media

b- Adding 1 ml of water sample in second five tubes of single strength media

c- Adding 10 ml of water sample in last five tubes of double strength media

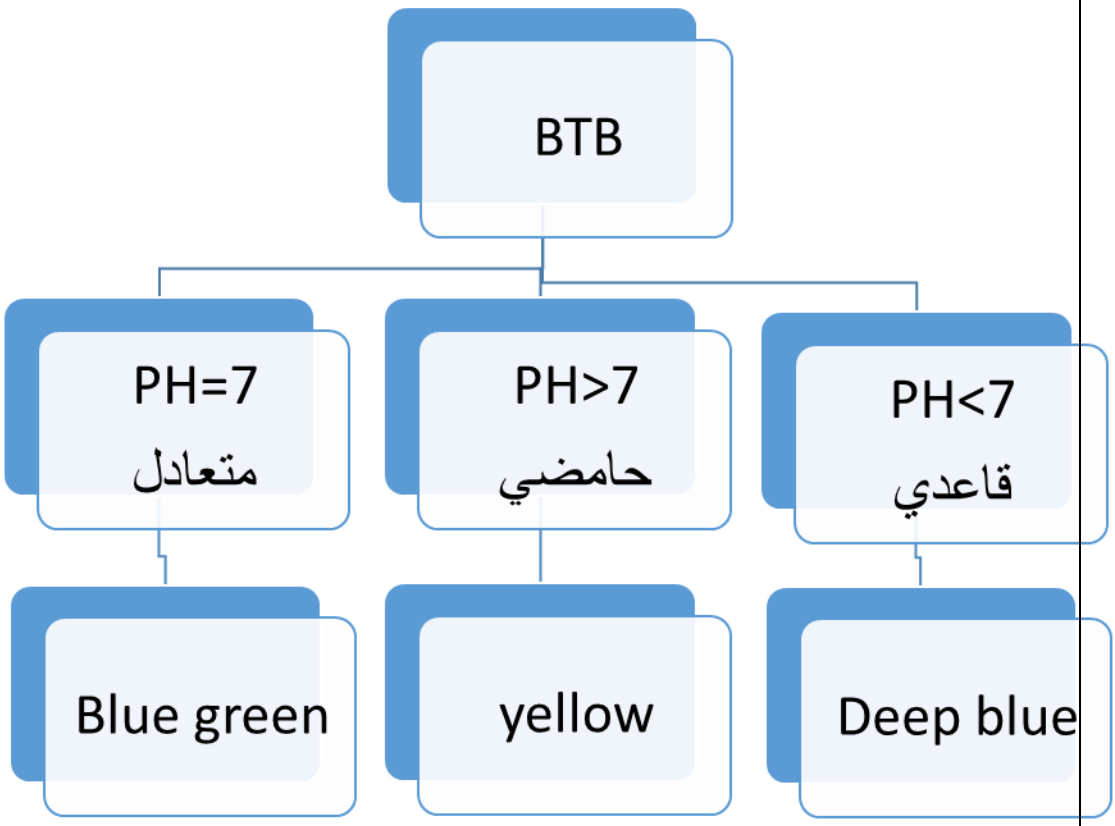
6-Incubate test tubes in incubator at 37^{o} C for 48 hr

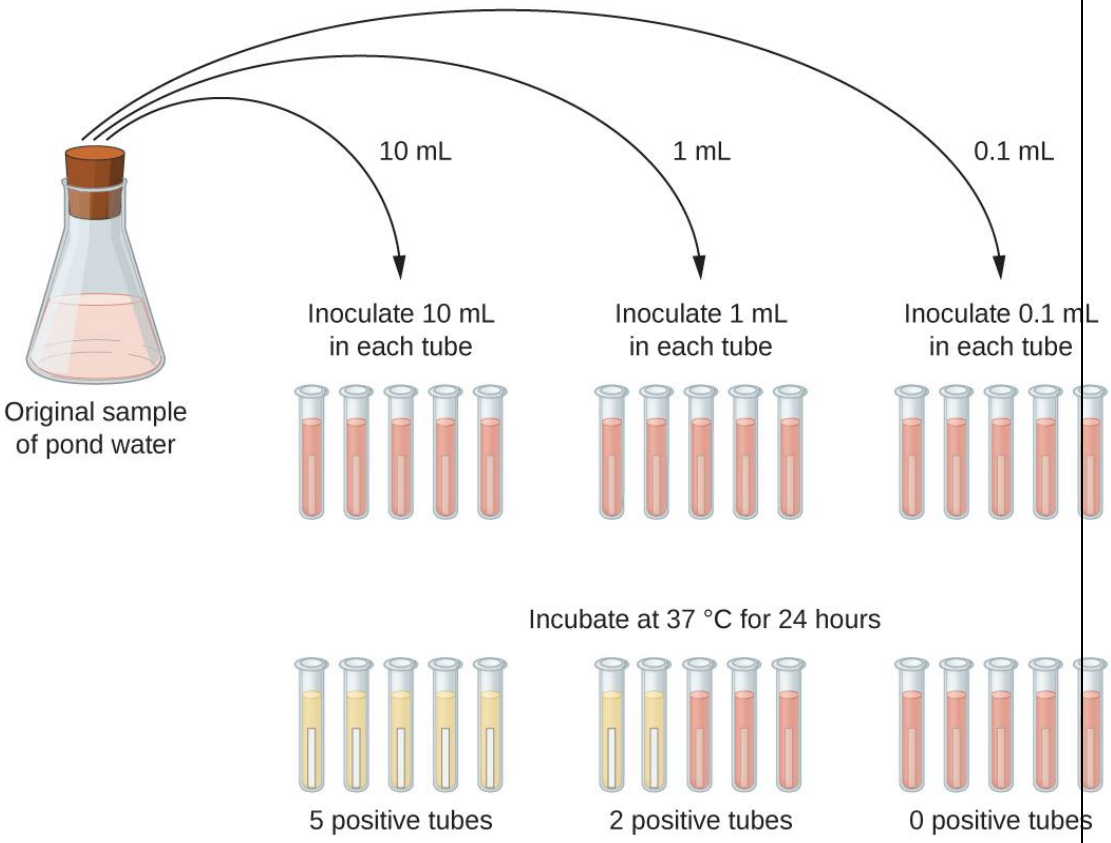
Why prepare single and double strength media? And why we duplicated the amount of nutrient?

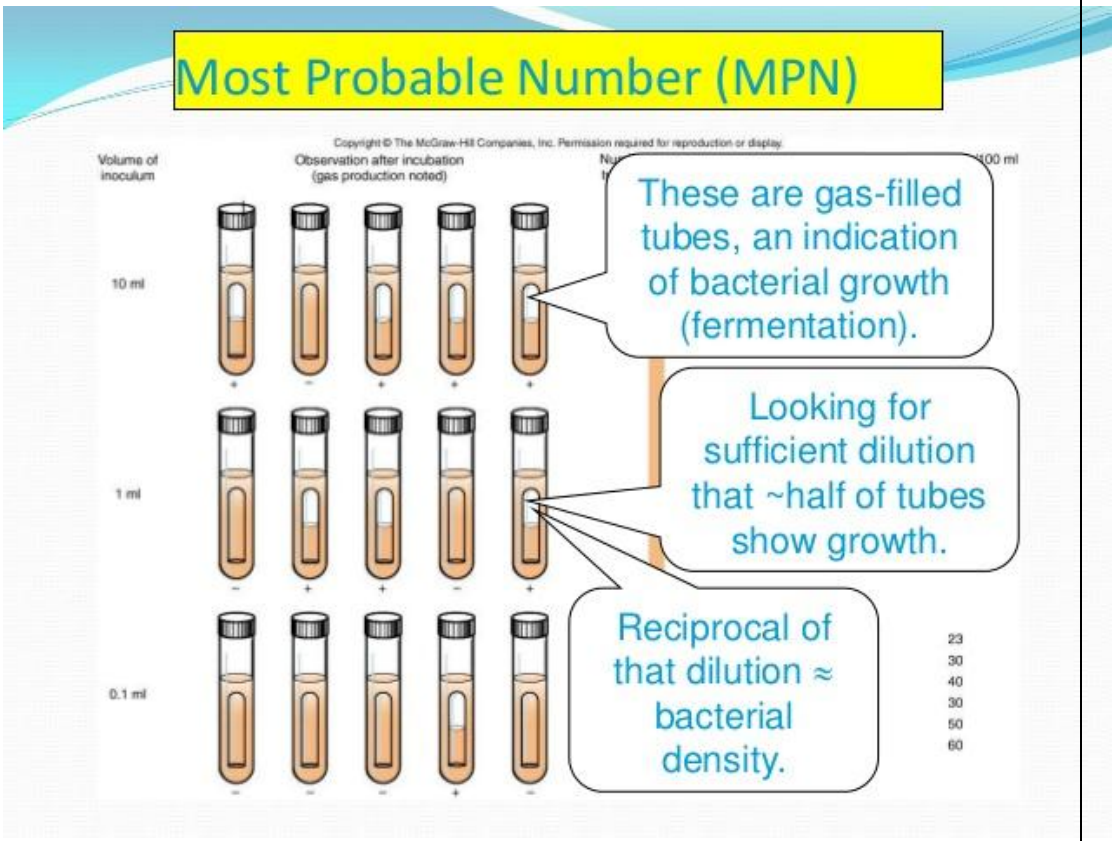
1- To make confirmation that bacteria found or not

2-Allow to the least amount of microbe to grow

3- Decrease the percentage of false in experiment




10 mL
1 mL
0.1 mL
Original sample
of pond water
Inoculate 10 mL
in each tube
Inoculate 1 mL
in each tube
Inoculate 0.1 mL
in each tube
Incubate at 37 °C for 24 hours
5 positive tubes
2 positive tubes
0 positive tubes



Observation:

1- Appearance of blue green colour.

2- Appearance of yellow colour.

Comment

1- Appearance of blue green colour indicate that water is not contaminated with bacteria this meaning water is qualified.

2- Appearance of yellow colour indicate that bacteria found on water and make fermentation process, produce an acid so medium become acidic and the colour of BTB is yellow in acidic medium this meaning water is non-qualified.

MPN values per 100 ml of sample and 95% confidence limits for various combinations of positive and negative results (when five 10-ml, five 1-ml and five 0.1 ml test portions are used)

No. of tubes giving a positive reaction :			MPN (per 100 ml)	95% confidence limits	
5 of 10 ml	5 of 1 ml	5 of 0.1 ml		Lower	Upper
0	0	0	<2	<1	7
0	1	0	2	<1	7
0	2	0	4	<1	11
1	0	0	2	<1	7
1	0	1	4	<1	11
1	1	0	4	<1	11
1	1	1	6	<1	15
2	0	0	5	<1	13
2	0	1	7	1	17
2	1	0	7	1	17
2	1	1	9	2	21
2	2	0	9	2	21
2	3	0	12	3	28
3	0	0	8	1	19
3	0	1	11	2	25
3	1	0	11	2	25
3	1	1	14	4	34
3	2	0	14	4	34
3	2	1	17	5	46

Confirmed test

Indicate the presence of Fecal coli

Procedure:

1- Prepare E.M.B media (Eosin methylene blue agar media) with autoclave sterilization

2- Pour E.M.B media in sterile petriplates and let plates to solidify in room temperature

3- Take inoculum from lowest conc. tubes (0.1ml)

In case of presumptive test why?

(to make the number of appearance colonies is few and it is easily to be counted)

4-Incubate petriplates in incubator at 37^{o} C for 48hr

- Observation

1- Appearance of green metallic colonies

2- Appearance of normal green colour colonies

Comment

1- Appearance of green metallic colonies indicate water contaminated by fecal coli bacteria.

2- Appearance of normal green colour colonies indicate water contaminated by E. coli bacteria.

Completed test

1- Prepare lactose broth media in test tubes single strength with autoclave sterilization

2- Inoculate tubes with metallic green colonies

3- incubate in incubator at37 for 48hr

Observation

Appearance yellow colour

Comment

appearance of yellow colour indicate that bacteria found on water and make fermentation process, produce an acid so medium become acidic and the colour of BTB is yellow in acidic medium this meaning water is non-qualified

Coagulase test

Introduction to Coagulase Test

Coagulase test is used to differentiate *Staphylococcus aureus* (positive) which produce the enzyme coagulase, from *S. epidermis* and *S. saprophyticus* (negative) which do not produce coagulase. i.e Coagulase Negative *Staphylococcus* (CONS).

Principle of Coagulase Test

Coagulase is an enzyme-like protein and causes plasma to clot by converting fibrinogen to fibrin. *Staphylococcus aureus* produces two forms of coagulase: bound and free.

Bound coagulase (clumping factor) is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in an alternation of fibrinogen so that it precipitates on the staphylococcal cell, causing the cells to clump when a bacterial suspension is mixed with plasma. This doesn't require coagulase-reacting factor.

Free coagulase involves the activation of plasma coagulase-reacting factor (CRP), which is a modified or derived thrombin molecule, to from a coagulase-CRP complex. This complex in turn reacts with fibrinogen to produce the fibrin clot.

Procedure and Types of Coagulase Test

Slide Test (to detect bound coagulase)

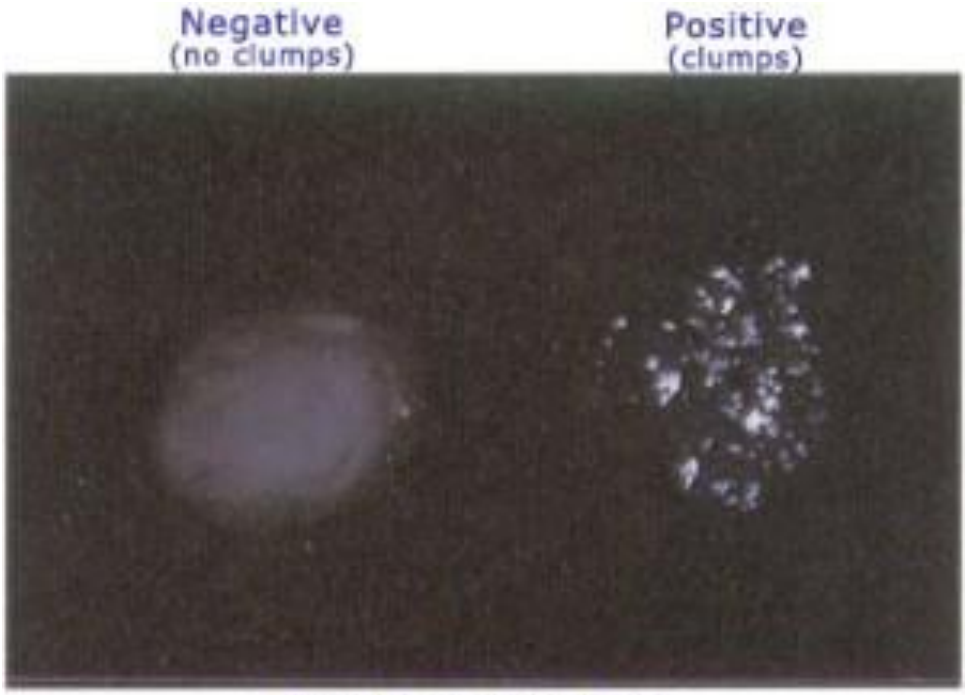
1. Place a drop of physiological saline on each end of a slide, or on two separate slides.
2. With the loop, straight wire or wodden stick, emulsify a portion of the isolated colony in each drops to make two thick suspensions.
3. Add a drop of human or rabbit plasma to one of the suspensions, and mix gently.
4. Look for clumping of the organisms within 10 seconds.
5. No plasma is added to the second suspension to differentiate any granular appearance of the organism from true coagulase clumping.

Tube Test (to detect free coagulase)

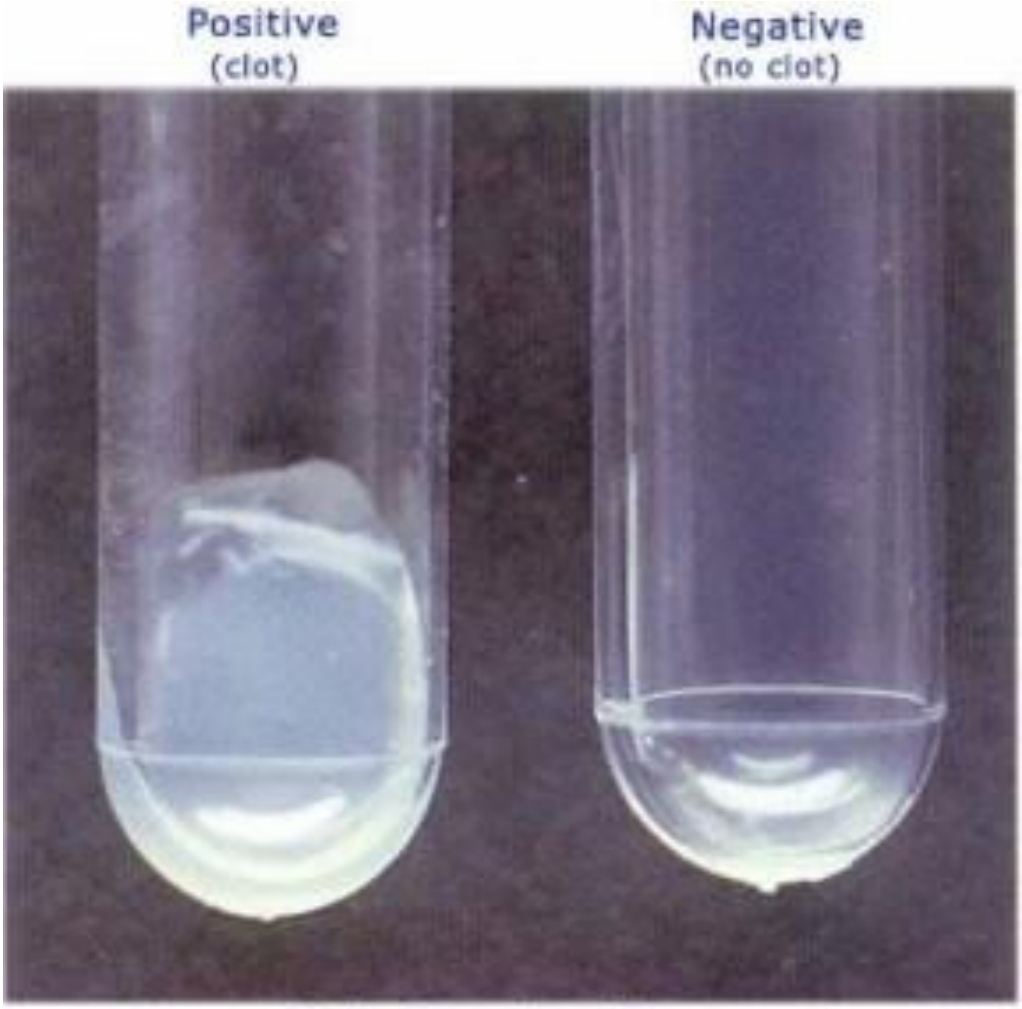
1. Dilute the plasma 1 in 10 in physiological saline (mix 0.2 ml of plasma with 1.8 ml of saline).
2. Take 3 small test tubes and label as T (Test), P (Positive Control) and N (Negative Control). Test is 18-24-hour broth culture, Positive control is 18-24 hr S. aureus broth culture and Negative control is sterile broth.
3. Pipette 0.5 ml of the diluted plasma into each tube.

4. Add 5 drops (0.1 ml) of the Test organisms to the tube labelled “T”, 5 drops of S. aureus culture to the tube labelled “P” and 5 drops of sterile broth to the tube labelled “N”.
5. After mixing, incubate the three tubes at 35-37 Degree Celsius.
6. Examine for clotting after 1 hours. If no clotting has occurred, examine at 30 minutes' intervals for up to 6 hours.

Interpretation of Coagulase Test



Slide Coagulase Test



Tube Coagulase Test

Fibrin Clot of any size- Positive
No Clot- Negative

- Clumping in both drops of slides indicates that the organism auto agglutinates and is unsuitable for the slide coagulase test. All the negative slide test must be confirmed using the tube test.
- During slide test, there may be chance to false positive results in case of citrate utilizing bacteria

(*Enterococcus* and *Pseudomonas*). In this case also, tube test should be performed and confirmed.

Examples

Coagulase Positive Organisms: *Staphylococcus aureus* and other animal host bacteria like *S. pseudintermedius, S. intermedius, S. schleiferi, S. delphini, S. hyicus, S. lutrae, S. hyicus*

Coagulase
Negative Organisms: *Staphylococcus epidermidis, S. saprophyticus, S. warneri, S. hominis, S. caprae*, etc.

Det of conc of catalyase enzyme

→ Catalyase: enzyme Produced from aerobic bacteria to convert H_2O_2 into H_2O and

The final receptor for aerobic bacteria is O_2 but in case of anaerobic bacteria is any receptor except O_2

$$H_2 \rightleftharpoons 2H^+ + 2\acute{e}$$

$$2e^- + O_2 \rightleftharpoons 2\bar{o}$$

$$2H^+ + 2O^- \rightleftharpoons H_2O_2$$ (hydrogen Peroxid خوخ آكسيد الهيدروجين)

⇒ H_2O_2 is toxic to bacterial cell so to remove its toxicity from it, bacteria Produce Catalyase enzyme, in case of aerobic bact

$$2H_2O_2 \xrightarrow{\text{Catalyase}} 2H_2O + O_2\uparrow$$

→ In case of anaerobic bacteria, the final receptor is not O_2 so it can't Produce Catalyase enzyme

Page :

Procedures:-

1- Prepare two test tubes one of them is control tube and the other is sample Tube or (Test Tube)

Control Tube contains 2mL H_2O_2, 2mL buffer soln, 2mL bacterial suspension

and sample Tube contain 2mL H_2O_2, 2mL buffer soln, 2mL dist H_2O

2- Put The two tubes in Water Path at 40 C° For 20min why?

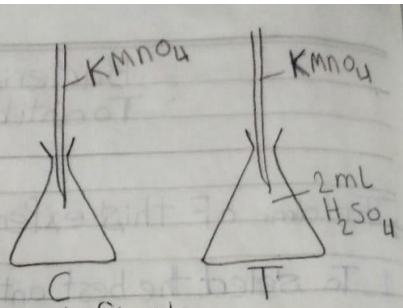
togive achance for bacteria to graw

3- Take the component of each tubes (Control and sample tube) and Put them in Conikal Flask

4- then titrate with $KMnO_4$ (0.5N) بورمنجنات البوتاسيوم and Put 2mL of H_2SO_4 in case of Test (Sample) Conical Flask to Stop the reactio between Catalyase and H_2O_2

The change of colour (end Point):

Colourless ⟶ Violet



Calculations :-

→ in Case of Control Conical flask

V_1 (Consumed Volume) ≡ Total hydrogen Peroxide H_2O_2

→ In Case of test Conical Flask

V_2 = un Consumed or unreacted Volume of H_2O_2

∴ V_3 (reacted H_2O_2) = V_1 (total H_2O_2) − V_2 (un Consumed)

∴ Catalyase Conc

$N.V_{[KMnO_4]} \equiv N.V_{[H_2O_2]}$

$0.5 \times V_3 = N \times 2$

$N_{(H_2O_2)} = \frac{0.5 \times V_3}{2}$

Conc of catalyase = $N \times \frac{\text{eq.wt}}{\frac{2\times1+2\times16}{2}}$ = ... g/L

Bacterial sensitivity to antibiotic

The aim of this experiment

1-To select the best antibiotic for bacteria.

2-To select the best antibiotic conc for bacterial cell

3-To detect or determine MIC and MBC of antibiotic

MBC: minimum bacteriostatic conc

MBC: minimum bacteriocidal conc.

MIC: is the higher dilution or lower conc. of antibiotic that inhibit the bacterial growth

MBC: is the higher dilution or lower conc. of antibiotic that kill bacterial cell

Procedures:

To detect the previous aims we use two methods

A) Disk method

B) Broth method

A) Disk method

Steps

1-prepare N.A media or muller Hinton media with auto clave sterilization

Nutrient agar media: 5g beef extract, 5g peptone, 5g Nacl ,15-20g agar / 1L dist H2O

Muller Hinton media: 2g beef extract ,17.5g peptone, 1.5g starch ,15-20g agar /1L dist H2O

2-Pour media in sterile Petri plates and let it to solidify in room temp

3-Inoculate plates by bacterial strain by 0.1ml of bacterial suspension and spread it on all the surface of plate

Bacterial suspension:

Sterile saline soln (0.9% NaCl)

Sterile dist. water (100 ml)

then take inoculum of bacterial growth and put it on sterile saline or sterile dist. water solution till form turbidity

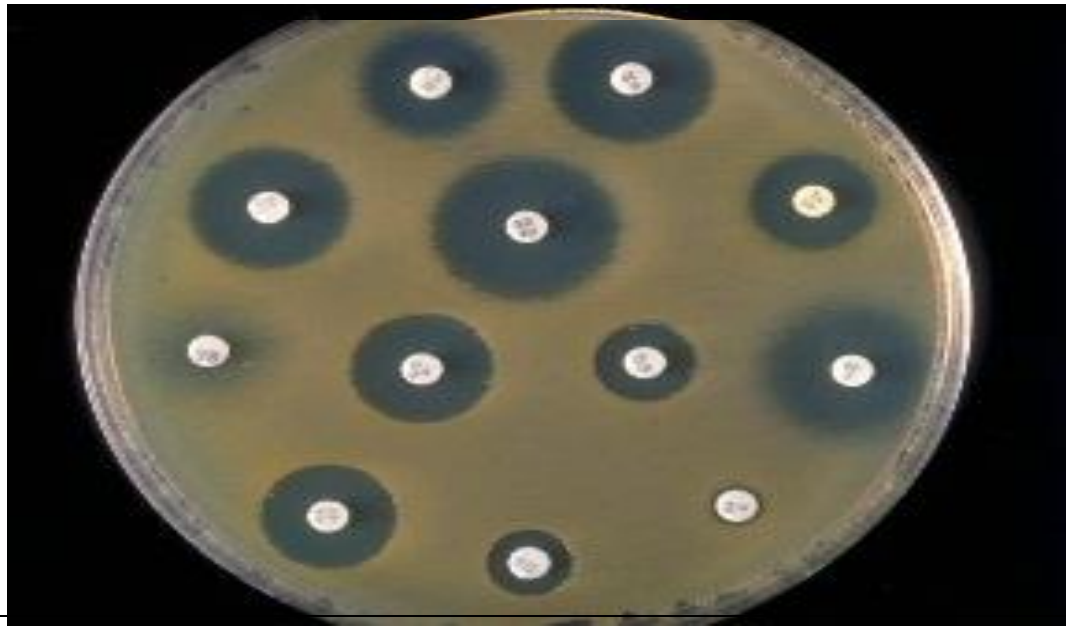
4-To detect the best antibiotic make soln. of different antibiotics

Antibiotic solution: by putting 1g per 100 ml sterile dist. water or putting 0.1 g per 10 ml dist. water

Then immerse sterile filter paper discs in antibiotic solution till saturated with antibiotic

5- Using sterile forceps put them on plate surface

6-Incubate plates in incubator at 37˚c for 48 hr



Observation

1-appearance of clear zone (bacteria sensitive to antibiotic)

2-doesn’t appearance of clear zone (bacteria resistance to antibiotic)

To detect the sensitivity of bacteria by clea rzone measure width of clear zone

For example:

Antibiotic	Resistance	Intermediate	Sensitive
penicillin	2-4mm	4-10 mm	10-20mm

Detection of the best conc. of the best antibiotics

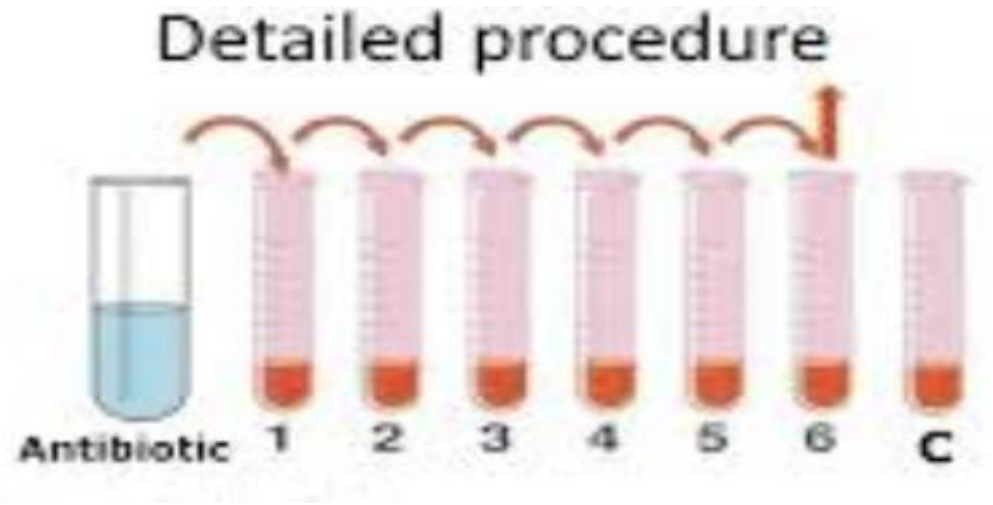
1-Make serial dilution of antibiotic causing sensitive

2-Immerse discs of filter paper in each tube

3- using sterile forceps put discs on the surface of plates

4- Incubate plates at incubator

The best conc. is the lowest conc. that cause kill of bacteria (no growth of bacteria)



B) Broth media (detection of MIC and MBC)

1-Prepare N.b media or muller Hinton broth in test tubes with autoclave sterilization.

2-Inoculate test tubes with bacterial strain (0.1 ml or 0.5 ml of bacterial suspension)

3-To detect the MIC of antibiotic make serial dilution of antibiotic for example: penicillin effect on staph aureus

4-Incubate tubes in incubator at 37℃ for 48 hr

If antibiotic effect on bacteria it makes media clear.

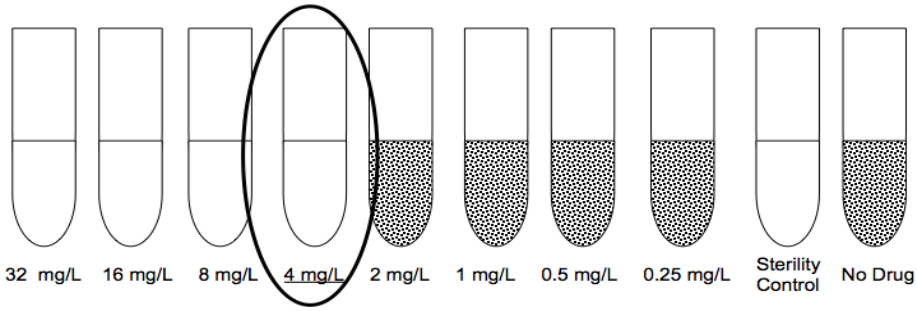
if antibiotic doesn't effect on bacteria, bacteria grow and the growth of bacteria detected by appearance of turbidity.

if there is turbidity in tube with conc. 2,1,0.5,0.25 mg/L

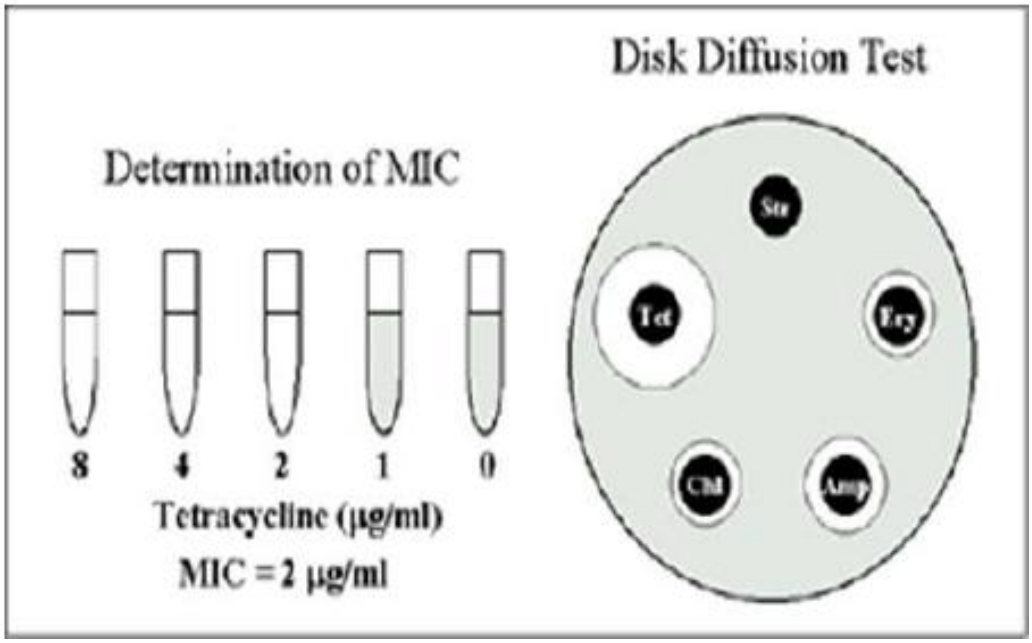
The MIC = 4 mg/L

Minimal Inhibitory Concentration (MIC) of Ampicillin for *E. coli*

1 mg/L=1 μg/ml



- Serial 2-fold dilutions of ampicillin in Mueller-Hinton broth
- Inoculum $5X10^5$ CFU/ml (clear, non-turbid); Incubate 18 hrs. @35º C.
- Assess turbidity, visually
- Concentration of drug in first tube without visible turbidity is the MIC (4 mg/L)



Determination of MBC

1- Prepare Petri plate with N.A or M.H agar media

2-Take 0.1 ml of MIC tube and inoculate it on the Petri plates.

3-incubation in incubator at 37℃ for 48 hr

Observation

NO growth of bacteria appears, antibiotic kill all bacteria (bactericidal effect)

i.e. MBC=MIC

Bacteria grow on media, antibiotic doesn't kill all bacteria (bacteriostatic effect)

i.e. MBC more than MIC

MBC may equal to MIC or more than MIC