V.V.VANNIAPERUMAL COLLEGE FOR WOMEN

VIRUDHUNAGAR

LAB MANUAL

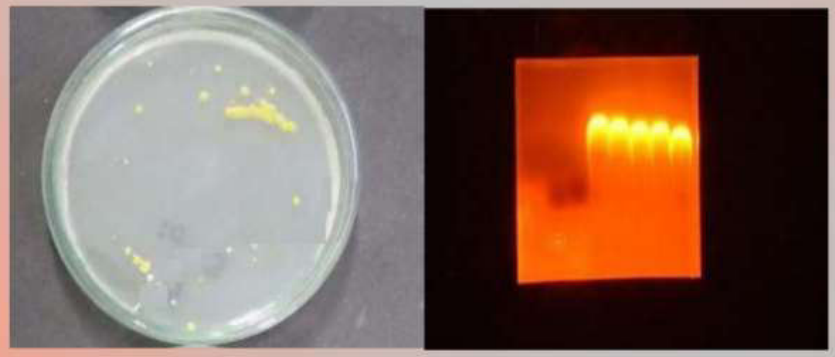
MICROBIOLOGY & BIOTECHNOLOGY

(UNDER DBT STAR COLLEGE SCHEME)

No HRD-11011/163/2020-HRD-DBT

Department of Biotechnology, Ministry of Science and Technology

MHRD, New Delhi



Dr.P.VEERAMUTHUMARI

Assistant Professor of Zoology

V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

V.V.VANNIAPERUMAL COLLEGE FOR WOMEN

(Belonging to Virudhunagar Hindu Nadars)
An Autonomous Institution Affiliated to Madurai Kamaraj University
Re-accredited with 'A' Grade (3rd cycle) by NAAC
VIRUDHUNAGAR – 626 001 (TAMIL NADU)

DBT STAR COLLEGE SCHEME

Department of Biotechnology, Ministry of Science and Technology
Government of India, New Delhi

Chairman & Principal : Dr.(Tmty.) S.M. MEENA RANI, M.Sc.,M.Phil.,P.G.D.C.A.,Ph.D.,
Contact number: 9498088703
e-mail:smmeenarani@gmail.com

Coordinator & Member Secretary : Dr.(Tmty.) M.TAMILSELVI, M.Sc.,M.Phil.,Ph.D.,
Contact Number:9894883106
e-mail:tamilasc.selvi08@gmail.com

FOREWORD

The purpose of writing this Lab Manual on "Microbiology and Biotechnology" is to provide standard procedure for the new practicals included in the updated curriculum under **Star College Scheme. Department of Biotechnology, Ministry of Science and Technology, New Delhi** for strengthening UG Science Departments and for promoting teaching, learning and Research. The hands-on learning experience in a laboratory helps the students easier to understand challenging theories and concepts.The skills developed by the students will play a significant role in future to perform various experiments and to conduct many laboratory tests in projects and researches.The laboratory experiments carried out in a laboratory strengthen the key concepts of the subjects that have learnt in their theory classes and it gives them a golden opportunity to apply their knowledge in research, inturn invention. They could learn essential experimental techniques that are necessary for their career to become an effective biologist in the field of research.

The basic practicals in microbiology such as Gram staining, culture techniques, biochemical test for identification of economically and commercially important microbes and advanced instruments will be useful to explore the microbial diversity of an environment. The included practicals will help the students to gain in-depth knowledge about the theoretical concept in the classroom. This manual also helps the students to develop the technical skills in doing experiments and handling advanced instruments.

The laboratory techniques in biotechnology play a key role in the future career of students as researchers. The experiments in biotechnology will help the students to meet the challenges in the environment and society in reducing environmental pollution, providing nutritional supplements and in maintaining the fertility of soil to improve the agricultural yield and thereby promoting the economy of the Nation.

We, **the Department of Zoology**, **the Coordinator**, **the Principal** and **the Management** of **V.V.Vanniaperumal College for Women, Virudhunagar** thank the **Memebers of DBT, Ministry of Science and Technology, New Delhi** for providing financial assistance for the benefits of the student community.

MEMBER SECRETARY / COORDINATOR **CHAIRMAN / PRINCIPAL**

V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

MICROBIOLOGY

BACTERIAL GROWTH CURVE

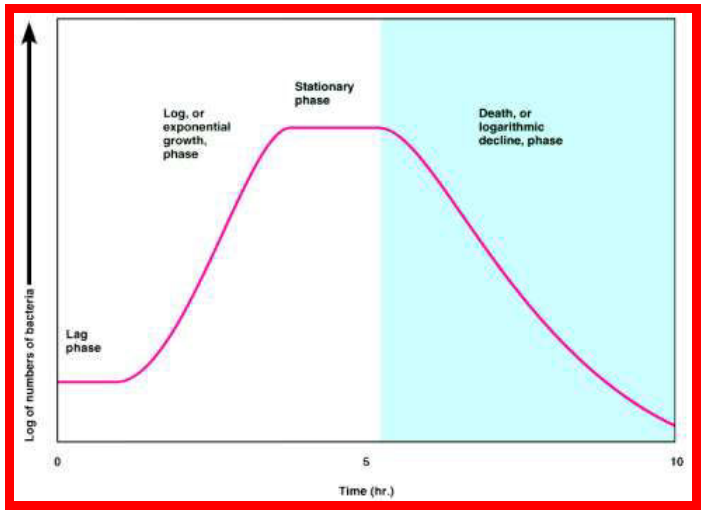


Figure: A Typical Growth Curve for the Bacterial Strain

V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

BACTERIAL GROWTH CURVE

AIM:

To study the different phases of bacterial growth in the broth culture medium.

MATERIALS REQUIRED:

A]	**EQUIPMENT**	:	Incubator, Shaker and Spectrophotometer
B]	**APPARATUS & GLASSWARE**	:	Conical flasks, Measuring cylinder, Micropipettes, Sterile test tubes, Sterile Petriplates, Sterile Loops and Tips.
C]	**CHEMICALS**	:	Luria Bertani Broth (LB) and Nutrient Broth
D]	**BACTERIAL CULTURE**	:	*E.coli*

PRINCIPLE

Bacteria need specific medium for its growth. When bacteria are inoculated into a liquid culture medium and the cell population is counted at intervals, it is possible to plot a typical bacterial growth curve that shows the growth of cells over time. It shows four distinct phases of growth.

Lag phase: Slow growth or lack of growth due to physiological adaptation of cells to culture conditions or dilution of exoenzymes due to initial low cell densities.

Log or exponential phase: Optimal growth rates, during which cell numbers double at discrete time intervals known as the mean generation time.

Stationary phase: Growth (cell division) and death of cells counterbalance each other resulting in no net increase in cell numbers. The reduced growth rate is usually due to a lack of nutrients and/or a buildup of toxic waste constituents.

Decline or death phase: Death rate exceeds growth rate resulting in a net loss of viable cells.

Turbidimetric determination is useful for plotting growth curves of bacteria in broth or liquid media. It is one of the simplest methods used to analyze trends in growth because it uses a spectrophotometer to track changes in the optical density (OD) over time. In other words, as the number of cells in a sample increases, the transmission of light through the sample will decrease.

PROCEDURE:

DAY : 1

1. Using sterile loop, streak a loopful of bacterial culture onto the agar plate.
2. Incubate at 37ºC for 18-24 hours.

DAY : 2

3. Pick up a single colony of a specific strain from the agar plate and inoculate it into a test tube containing 10 ml of autoclaved broth.
4. Incubate the test tube overnight at 37ºC.

DAY : 3

5. Take 250 ml of autoclaved broth in a sterile 500 ml conical flask.
6. Inoculate 5 ml of the overnight grown culture in above flask.
7. Take OD at zero hour. Incubate the flask at 37ºC.
8. Aliquot 1 ml of the culture suspension at an interval of every 30 minutes and take the optical density (OD) at a wavelength of 600 nm using spectrophotometer, till the reading becomes static. Alternatively, 50-100 µl of formaldehyde is added to all the 1ml aliquots of culture suspension taken after every 30 minutes. Optical density of all the aliquots is taken at the end of the experiment.
6. At the end of experiment, plot a graph of time in minutes on X axis versus optical density at 600nm on Y axis to obtain a growth curve of bacteria.

RESULT:

A logarithmic growth curve is obtained showing the changes in size of a bacterial population over time in the culture. The growth curve is hyperbolic due to exponential bacterial growth pattern. Bacterial growth occurs in four distinct phases over time.

NUMBER OF BENEFICIARIES: 38

OUTCOME:

Students learnt the growth phases of bacterial strain. They are also able to draw the growth curve for the specific bacterial strain against the time factor. They can be able to use it for industrial and agricultural applications.

GRAM STAINING TECHNIQUE FOR IDENTIFICATION OF BACTERIA

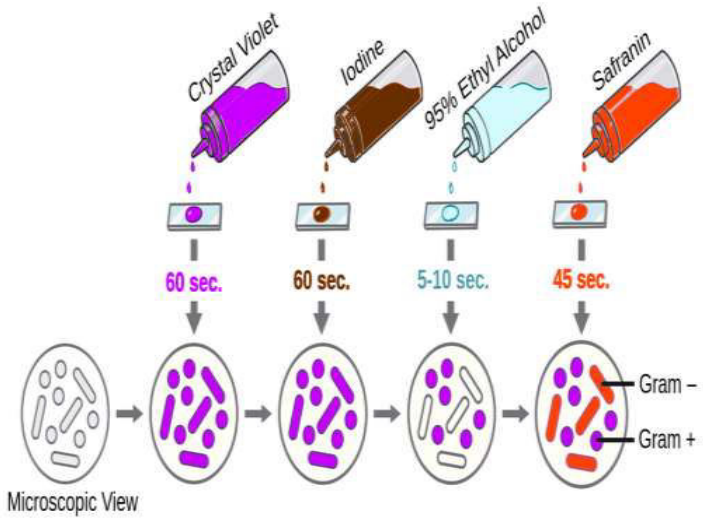


Figure : The Sequence of Gram Staining Technique

GRAM STAINING TECHNIQUE FOR IDENTIFICATION OF BACTERIA

AIM:

To identify and differentiate between the two major categories of bacteria Gram positive and Gram negative.

MATERIALS REQUIRED:

A]	**EQUIPMENT**	:	Microscope
B]	**APPARATUS & GLASSWARE**	:	Watch glass, Microscopic slide, pipette, Tips and Sterile Loops.
C]	**CHEMICALS**	:	Broth Crystal violet (primary stain), Iodine solution/Gram's Iodine, Decolorizer (e.g. ethanol), Safranin (secondary stain) and Water,
D]	**BACTERIAL CULTURE**	:	*E.coli* and *Lactobacilli sp.*

PRINCIPLE

The two major groups of bacteria can be divided into gram-positive and gram-negative. The Gram stain technique is based on the differential structure of the cellular membranes and cell walls of the two major groups of bacteria. Gram-positive organisms contain a highly cross-linked layer of peptidoglycan that retains the primary dye, crystal violet (CV), following the application of the mordant, iodine (I). The iodine and crystal violet form a complex within the peptidoglycan. When decolorizer is applied to the cells, the CV-I complex remains within the cell, making it appear dark purple to blue. The gram-negative organisms do not contain a thick cross-linked layer of peptidoglycan. The peptidoglycan is loosely distributed between the inner cell and the outer cell membranes. Following the application of the crystal violet and iodine, the CV-I complexes are not trapped within the peptidoglycan. Application of the acid-alcohol decolorizer dehydrates the outer cellular membrane, leaving holes in the membrane and effectively washing or removing the CV-I complex from the cells. The cells appear colorless. To make the colorless cells visible, a secondary stain, safranin, is applied, leaving the gram-negative cells pink.

PROCEDURE:

1. Take a clean, grease free slide.
2. Prepare the smear of suspension on the clean slide with a loopful of sample.
3. Air dry and heat fix
4. Add Crystal Violet on heat fixed sample and keep it for about 30 seconds to 1 minute and rinse with water.
5. Flood the gram's iodine for 1 minute and wash with water.
6. Then, wash with 95% alcohol or acetone for about 10-20 seconds and rinse with water.
7. Add safranin for about 1 minute and wash with water.
8. Air dry, Blot dry and Observe under Microscope.

RESULTS:

The given bacterial sample is Gram positive / Gram negative.

Gram-positive: Blue/Purple Color

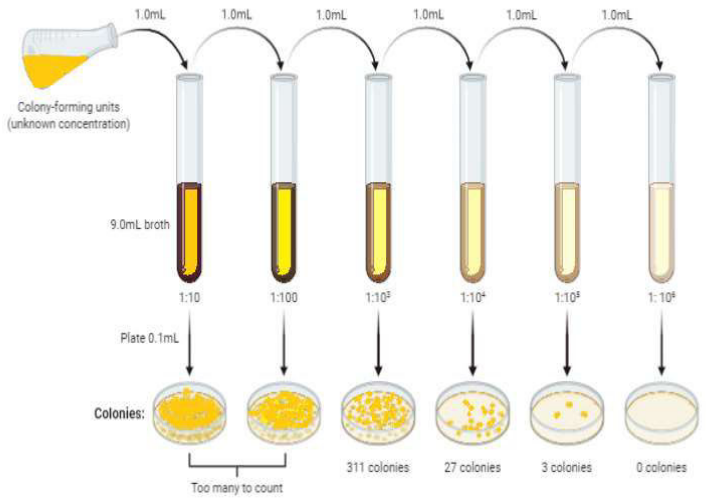
Gram-Negative: Red/Pink Color

NUMBER OF BENEFICIARIES: 38

OUTCOME:

Students are able to learn the technique on Gram staining and become proficient at performing the Gram stain consistently and accurately.

PURE CULTURE TECHNIQUES - SERIAL DILUTION TECHNIQUE



PURE CULTURE TECHNIQUES - SERIAL DILUTION TECHNIQUE

AIM:

To enumerate the number of colonies cultured from serial dilutions of the sample.

MATERIALS REQUIRED:

A]	**EQUIPMENT**	:	Incubator
B]	**APPARATUS & GLASSWARE**	:	Sterile 1.5 ml tubes, Sterile Petri plates, pipette tips and micropipettes.
C]	**CHEMICALS**	:	Sterile dilution medium and Nutrient broth.

PRINCIPLE:

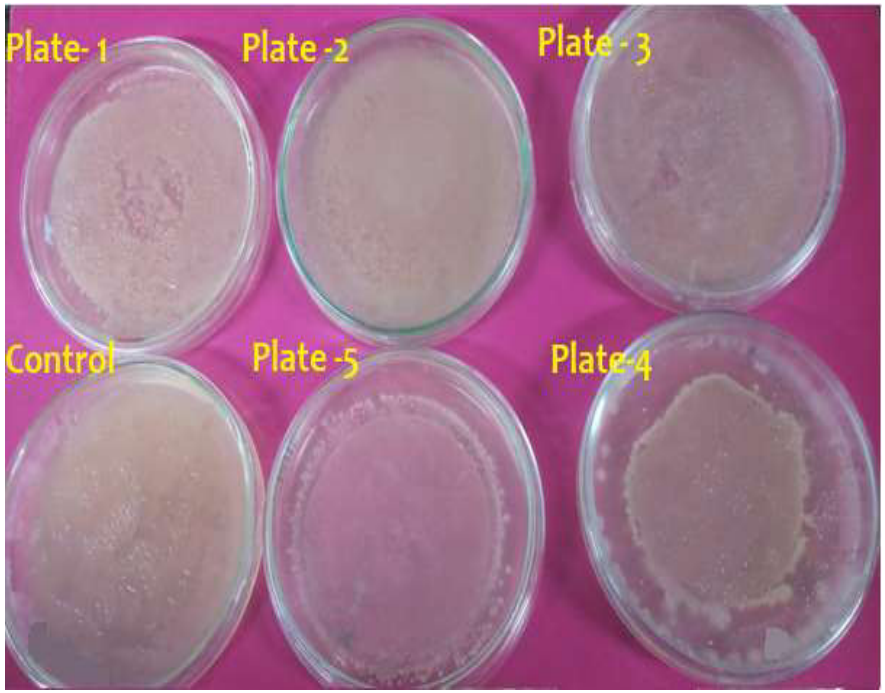
In serial dilution, the density of cells is reduced in each step so that it is easier to calculate the concentration of the cells in the original solution by calculating the total dilution over the entire series. Serial dilutions are commonly performed to avoid having to pipette very small volumes (1-10 µl) to make a dilution of a solution. By diluting a sample in a controlled way, it is possible to obtain incubated culture plates with an easily countable number of colonies (around 30–100) and calculate the number of microbes present in the sample by applying the following formula.

$$\text{Serial dilution formula/calculations} = \frac{\text{Volume of sample}}{\text{Volume of sample + Volume of diluent}}$$

For a ten-fold dilution, 1 ml of sample is added to 9 ml of diluent. In this case, the dilution factor for that test tube will be

$$\text{Dilution factor} = \frac{1\text{ ml}}{1\text{ ml} + 9\text{ml}} = \frac{1}{10} = 10^{-1}$$

SERIAL DILUTION CULTURE PLATES



After the first tube, each tube is the dilution of the previous dilution tube.

Now, for total dilution factor,

Total dilution factor for the second tube = dilution of first tube × dilution of the second tube.

EXAMPLE:

For the first tube, dilution factor = 10^{-1} (1 ml added to 9 ml)

For the second tube, dilution factor = 10^{-1} (1ml added to 9 ml)

Total dilution factor = previous dilution × dilution of next tube

= total dilution of $10^{-1} \times 10^{-1} = 10^{-2}$

PROCEDURE

The following is the procedure for a ten-fold dilution of a sample to a dilution factor of 10^{-6}:

1. The sample/culture is taken in a test tube and six test tubes, each with 9 ml of sterile diluents, which can either be distilled water or 0.9% saline, are taken.
2. A sterile pipette is taken.
3. 1 ml of properly mixed sample/culture is drawn into the pipette.
4. The sample is then added to the first tube to make the total volume of 10 ml. This provides an initial dilution of 10^{-1}.
5. The dilution is thoroughly mixed by emptying and filling the pipette several times.
6. The pipette tip is discarded, and a new pipette tip is attached to the pipette.
7. Now, 1 ml of mixture is taken from the 10^{-1} dilution and is emptied into the second tube. The second tube now has a total dilution factor of 10^{-2}.
8. The same process is then repeated for the remaining tube, taking 1 ml from the previous tube and adding it to the next 9 ml diluents.
9. As six tubes are used, the final dilution for the bacteria/cells will be 10^{-6} (1 in 1,000,000).

RESULT:

In serial dilution, the cell count or density gradually decreases as the serial number increases in each step. Single colony was observed in plate-5.

NUMBER OF BENEFICIARIES: 38

OUTCOME: Students learnt the techniques on reducing the bacterial population from different samples (Food/Soil/Water/Biological samples)

PURE CULTURE TECHNIQUE - POUR PLATE METHOD

V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

PURE CULTURE TECHNIQUE - POUR PLATE METHOD

AIM:

To isolate the pure culture strain and count the number of viable organisms in a liquid such as water, milk, urine or broth culture.

REQUIREMENTS:

A]	**EQUIPMENT**	:	Incubator
B]	**APPARATUS & GLASSWARE**	:	Sterile 1.5 ml tubes, Sterile Petri plates, pipette tips and micropipettes.
C]	**CHEMICALS**	:	Nutrient Agar Medium and Sterile Water.
D]	**BACTERIAL CULTURE**	:	24 hours old nutrient broth culture of two different organisms,

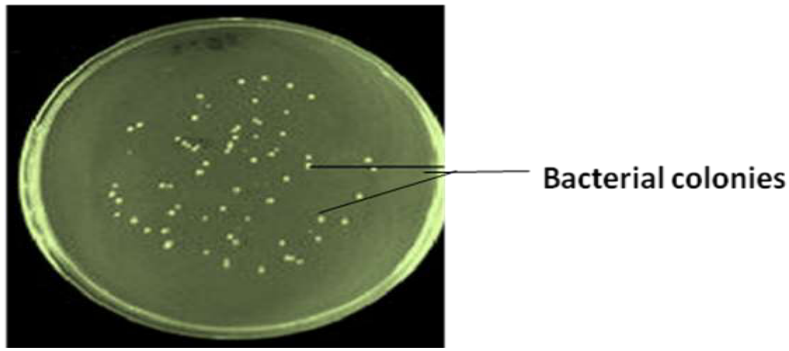
PRINCIPLE:

In this Method, serial dilutions of the inoculum (serially diluting the primary specimen) are added in sterile Petri plates to which melted and cooled (42-45°C) agar medium is poured and completely mixed by revolving the plates which are then left to solidify. After incubation, the plates are observed for the appearance of individual colonies growing everywhere in the medium. The pure colonies which are of varying size, shape and colour may be isolated/transferred into test tube culture media to prepare pure cultures.

PROCEDURE:

1. Place the 7 agar deep tubes within a boiling water bath for melting of agar.
2. Allow them to cool to 48°C.
3. Label the empty sterile culture tube as number 1 and the six water blanks (containing 9ml of water) as numbers 2 through 7 with a wax marking pencil. Also, label the Petri plates 1 through 7.
4. Now Place the labeled tubes in a test tube rack.
5. Mix well the 24 hours old broth culture to equally distribute the bacterial cells in the tube.
6. After mixing, remove the cotton plug and aseptically transfer 1 ml of the bacterial suspension from tube number 1 to water blank tube number 2 and return the pipette to tube number 1.

POUR PLATE METHOD



7. Shake the tube number 2 with a fresh sterile pipette and transfer 1 ml to tube number 3. Return the pipette to tube number 2.
8. Prepare serial dilutions till the 6 water blanks (number 2-number 7) are used by using fresh sterile pipettes each time by repeating step 7.
9. Transfer 1 ml of the bacterial suspension each from tube numbers 1 to 7 to Petri plates numbers 1 to 7 using respective pipettes.
10. Remove a nutrient agar tube from the water bath (at 45°C) and pour the medium into plate 1 and rotate the plate clockwise and anticlockwise gently to ensure uniform distribution of cells in the medium.
11. Repeat step 10 for the addition of medium to plates 2, 3, 4, 5, 6, and 7.
12. Allow the medium to solidify.
13. Incubate the inoculated plates for 24-48 hours at 37°C in an inverted position (lid on bottom).
14. The total number of colony-forming units (CFU) on the surface of an agar medium is enumerated.

RESULT:

The Calculation of CFU/ml is done by using the formula: CFU/ml= CFU * dilution factor * 1/aliquot (the volume of diluted specimen (aliquot) is either 0.1 or 1.0 ml).

NUMBER OF BENEFICIARIES: 38

OUTCOME:

Students learnt the techniques on isolating pure culture strain from liquid samples.

SPREAD PLATE METHOD

SPREAD PLATE METHOD

AIM:

To isolate the microorganisms in a mixed culture and to enumerate the bacteria in a solution.

MATERIALS REQUIRED:

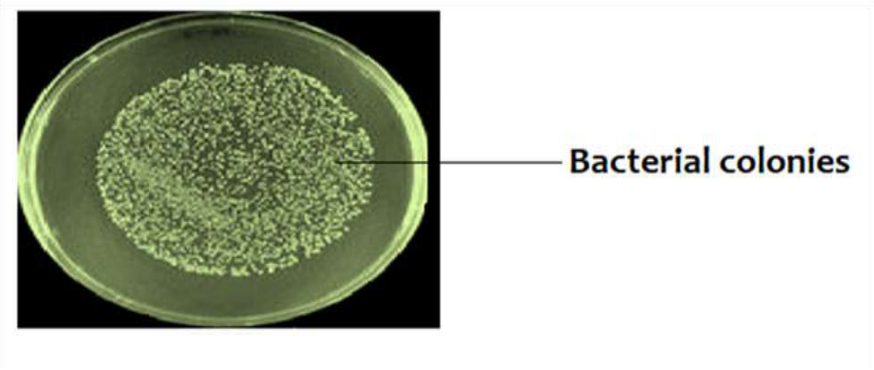
A]	**EQUIPMENT**	:	Incubator and Colony counter.
B]	**APPARATUS & GLASSWARE**	:	Beaker, Conical flaks, test tubes**, S**terile 1.5 ml tubes, Graduated pipette (1ml), glass rod spreader, Sterile Petri plates, pipette tips and micropipettes..
C]	**CHEMICALS**	:	Nutrient Agar Medium and Sterile Water.
D]	**BACTERIAL CULTURE**	:	24 hours old nutrient broth culture of two different organisms,

PRINCIPLE:

In this technique, a serially diluted specimen containing 2 or more bacteria or microbe (Mixed culture) is used which is spread over the solidified agar media plates as a thin layer with the help of a sterile L-shape glass rod (Spreader) while the media plate is spun on a turntable. The principle behind this method is that when the Media plate is spun, at some stage, single cells will be deposited with the bent glass rod (Spreader) onto the surface of the Agar media. Some of the cells present in the specimen / diluted specimen will be separated from each other by a distance sufficient to allow the colonies that develop to be free from each other.

PROCEDURE:

1. Make a dilution series from a sample.
2. Pipette out 0.1 ml from the appropriate desired dilution series onto the center of the surface of an agar plate.
3. Dip the L-shaped glass spreader into alcohol.
4. Flame the glass spreader (hockey stick) over a Bunsen burner.



SPREAD PLATE

5. Spread the sample evenly over the surface of agar using the sterile glass spreader, carefully rotating the Petridish underneath at the same time.
6. Incubate the plate at 37°C for 24 hours.
7. Calculate the CFU value of the sample. Once you count the colonies, multiply by the appropriate dilution factor to determine the number of CFU/mL in the original sample.

RESULT:

Isolated countable colonies evenly spread across the surface of the agar was observed after the incubation period and counted the number of colonies using a magnifying device. Once counted the colonies, multiply by the appropriate dilution factor to determine the colony-forming units (CFU) present per ml in the original sample. CFU/ml = (no. of colonies x dilution factor) / volume of culture plate.

For example, suppose the plate of the 10^6 dilution yielded a count of 130 colonies. Then, the number of bacteria in 1 ml of the original sample can be calculated as follows:

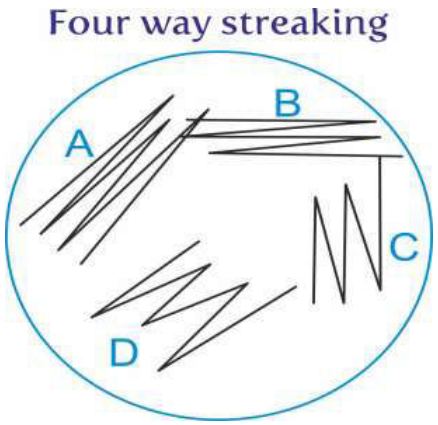
Bacteria/ml = (130) x (10^6) x 10 = 1.3 × 10^9.

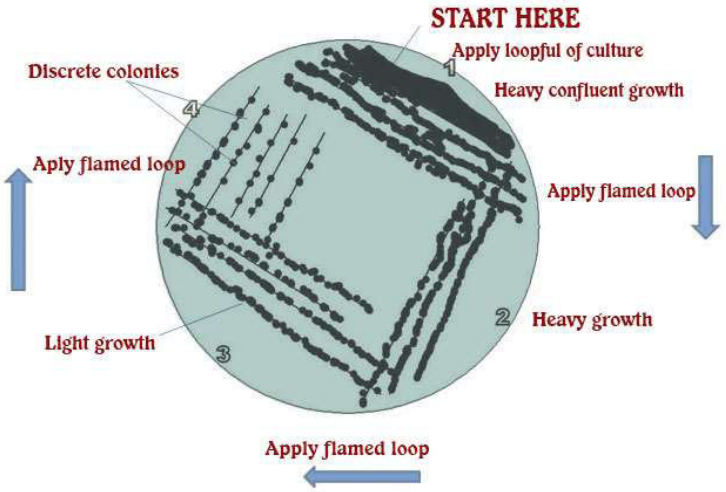
NUMBER OF BENEFICIARIES: 38

OUTCOME:

Students will be able to learn the technique of isolation of bacteria from a mixed culture.

STREAK PLATE METHOD





STREAK PLATE METHOD

AIM

To isolate the pure culture of bacteria from a mixed population of microorganisms.

MATERIALS REQUIRED:

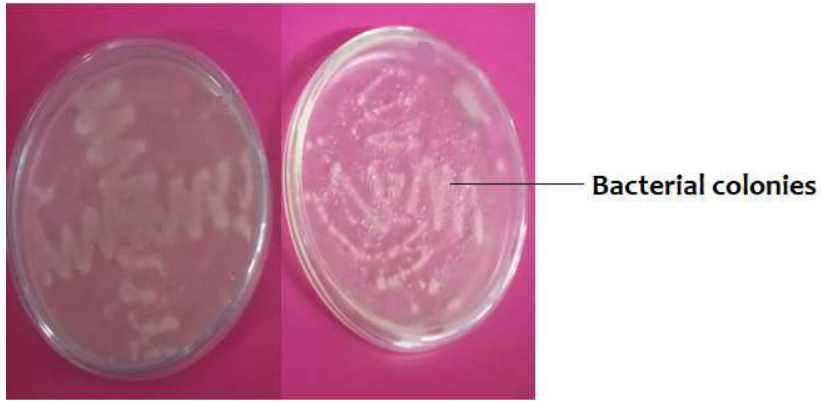
A]	**EQUIPMENT**	:	Incubator and Colony counter.
B]	**APPARATUS & GLASSWARE**	:	Beaker, Conical flaks, test tubes**, S**terile 1.5 ml tubes, Graduated pipette (1ml), glass rod spreader, Sterile Petri plates, pipette tips and micropipettes.
C]	**CHEMICALS**	:	Nutrient Agar Medium, Ethanol and Sterile Water.
D]	**BACTERIAL CULTURE**	:	24 hours old nutrient broth culture of two different organisms,

PRINCIPLE:

The isolation of pure cultures of microorganisms is a technique essential to many types of experiments in microbiology as well as in the identification of potential pathogens. One very common method to isolate bacteria and other microbes is by employing the streak plate technique. The sample/inoculum is diluted by streaking it across the surface of the agar plate. While streaking in successive areas of the plate, the inoculum is diluted to the point where there is only one bacterial cell deposited every few millimeters on the surface of the agar plate. When these lone bacterial cells divide and give rise to thousands and thousands of new bacterial cells, an isolated colony is formed. Pure cultures can be obtained by picking well-isolated colonies and re-streaking these on fresh agar plates.

PROCEDURE:

1. Sterilize the inoculating loop in the bunsen burner by putting the loop into the flame until it is red hot. Allow it to cool.



STREAK PLATE

2. Pick an isolated colony from the agar plate culture and spread it over the first quadrant (approximately 1/4 of the plate) using close parallel streaks or Insert the loop into the tube/culture bottle and remove some inoculum.
3. Immediately streak the inoculating loop very gently over a quarter of the plate using a back and forth motion.
4. Flame the loop again and allow it to cool. Going back to the edge of area 1 where just streaked, extend the streaks into the second quarter of the plate **(area 2).**
5. Flame the loop again and allow it to cool. Going back to the area just streaked (area 2), extend the streaks into the third quarter of the plate **(area 3).**
6. Flame the loop again and allow it to cool. Going back to the area that just streaked (area 3), extend the streaks into the center fourth of the plate **(area 4).**
7. Flame the loop once more.
8. The streaked plate incubated at 37ºC for 24 hours.

RESULT: Colonies grown in the agar plate was observed.

NUMBER OF BENEFICIARIES: 38

OUTCOME:

Students will be able to carry out the technique of isolation of bacteria from a mixed culture.

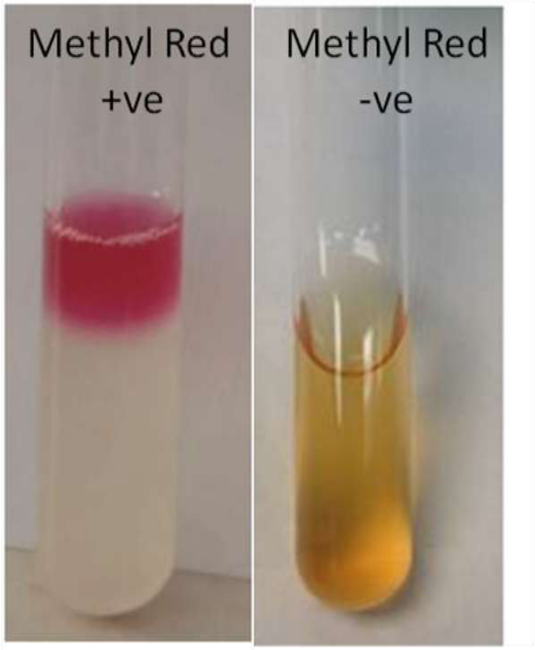
V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

BIOCHEMICAL TESTS

FOR

BACTERIAL IDENTIFICATION

METHYL RED TEST



V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

METHYL RED TEST

AIM: To check whether the organisms follow mixed acid fermentation pathway during their metabolism or not.

MATERIALS REQUIRED:

A]	**APPARATUS & GLASSWARE**	**:**	Test tubes, Glass rod spreader, pipette tips and micropipettes
B]	**CHEMICALS**	**:**	MR-VP broth and methyl red indicator
C]	**BACTERIAL CULTURE**	**:**	24 hours old nutrient broth culture of two different organisms *E.coli* and *Klebsiella.*

PRINCIPLE:

The basic principle of MR test is to check the ability of the organism to produce and maintain sufficient amount of stable acid as end product from glucose fermentation and to overcome the buffering capacity of the system.

Glucose ------→ 2 Pyruvate -------→ Succinic acid, Lactic acid, Acetic acid, Formic acid + CO_2 + H_2O

These acids lower the pH of the medium which is nearly neutral. The pH range of the methyl red indicator is 4.4-6. The production of acids lowers the pH of medium so the methyl red indicator changes it color to red but if there is no or low production of acids or production of other neutral end products, the pH of the medium remains fairly unchanged and the indicator shows yellow color. Hence, the red color of the broth medium after adding indicator is positive MR test. Enteric that subsequently metabolize pyruvic acid to neutral end products lower the pH of the medium to only 6.0. At this pH, methyl red is yellow, a negative test.

PROCEDURE:

1. Prepare MRVP broth in the test tubes.
2. Inoculate the broth aseptically with two loopful of respective bacteria culture.
3. Label the test tubes with name of organism inoculated.
4. Incubate the test tubes at 37ºC for 48-72hours.
5. Add few drops of methyl red indicator in the incubated tubes.
6. Observe the results.

RESULT:

Positive Reaction : A distinct red color indicated the presence of *E. coli*

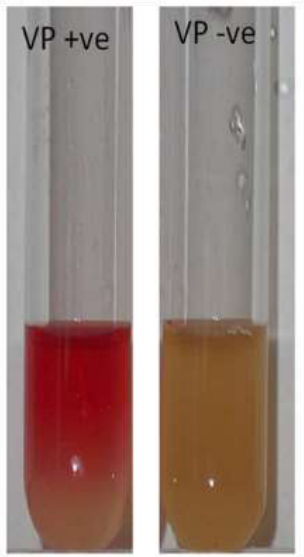
Negative Reaction: A yellow color indicated the presence of *Klebsiella pneumoniae*

NUMBER OF BENEFICIARIES: 38

OUTCOME:

The students can be able to distinguish members of the family Enterobacteriaceae and they are used to characterize other groups of bacteria including Actinobacteria.

VOGES-PROSKAUER TEST



V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

VOGES-PROSKAUER TEST

AIM:

To determine if an organism produces acetyl methyl carbinol from glucose fermentation.

PRINCIPLE:

Pyruvic acid, the pivotal compound in the fermentative degradation of glucose, is further metabolized through various metabolic pathways, depending on the enzyme systems possessed by different bacteria. One such pathway results in the production of acetoin (acetyl methyl carbinol)**,** a neutral-reacting end product. If present, acetyl methyl carbinol is converted to diacetyl in the presence of α-naphthol, strong alkali (40% KOH), and atmospheric oxygen. The diacetyl and guanidine-containing compounds found in the peptones of the broth then condense to form a pinkish-red polymer. Organisms such as members of the *Klebsiella, Enterobacter, Hafnia, Serratia* group produce **acetoin** as the chief end product of glucose metabolism and form smaller quantities of mixed acids. Acetoin reacts with VP reagent (α-napthol + KOH) to give pinkish red complex, which indicates positive test.

MATERIALS REQUIRED:

A]	**APPARATUS & GLASSWARE**	**:**	Test tubes, Glass rod spreader, pipette tips and micropipettes
B]	**CHEMICALS**	**:**	Methyl red-Voges-Proskauer (MR/VP) broth, α-napthol, Potassium hydroxide (40% KOH), Absolute alcohol and Deionized water.
C]	**BACTERIAL CULTURE**	**:**	24 hours old nutrient broth culture of two different organisms *E.coli* and *Klebsiella.*

PROCEDURE FOR VP TEST:

1. Inoculate a tube of MR/VP broth with a pure culture of the test organism.
2. Incubate for 24 hours at 35°C.
3. Inoculate the broth aseptically 2 loopful of respective bacterial culture
4. Label the test tubes with the name of the organism inoculated
5. Incubate the test tubes at 37°C for 48-72 hours.

6. Add 0.6mL of 5% α-naphthol*, followed by 0.2 mL of 40% KOH in the ratio 3: 1
7. Remove the cotton plug and shake the tubes for aeration.
8. Observe the results after 10-15 minutes.

RESULT:

VP positive: Pinkish red color at the surface indicated that presence of *Klebsiella* sp.

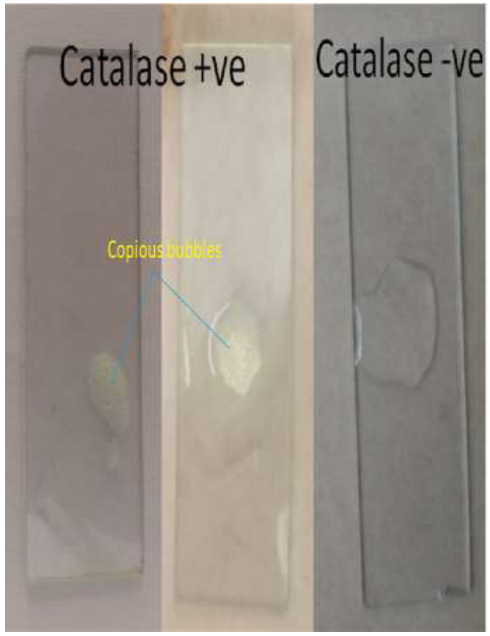
VP negative: Whitish yellow indicated that presence of *E.coli*.

NUMBER OF BENEFICIARIES: 38

OUTCOME:

The students can be able to distinguish between the members of the family Enterobacteriaceae and they are able to characterize other groups of bacteria including Actinobacteria.

CATALASE TEST



SLIDE METHOD

TEST TUBE METHOD

CATALASE TEST

AIM:

To analyse the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide (H_2O_2).

MATERIALS REQUIRED:

A]	APPARATUS & GLASSWARE	:	Test tubes, Glass rod spreader, pipette tips and micropipettes
B)	CHEMICALS	:	Hydrogen peroxide
C)	BACTERIAL CULTURE	:	Overnight cultured colonies

PRINCIPLE:

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old.

$$2H_2O_2 \xrightarrow[\text{Catalase}]{} 2H_2O + O_2\,(\text{Gas bubbles})$$

Bacteria thereby protect themselves from the lethal effect of Hydrogen peroxide which is accumulated as an end product of aerobic carbohydrate metabolism.

PROCEDURE :

TUBE METHOD

1. Pour 1-2 ml of hydrogen peroxide solution into a test tube.
2. Using a sterile wooden stick or a glass rod, take several colonies of the 18 to 24 hours test organism and immerse in the hydrogen peroxide solution.
3. Observe for immediate bubbling.

SLIDE METHOD

1. Few drops of hydrogen peroxide solution place on glass slide.
2. Using a sterile wooden stick or a glass rod, take several colonies of the 18 to 24 hours test organism and immerse in the hydrogen peroxide solution.

3. Observe for immediate bubbling.

RESULT

Positive : Copious bubbles produced, active bubbling

Negative: No or very few bubbles produced.

NUMBER OF BENEFICIARIES: 38

OUTCOME:

Students will be able to do the biochemical characterization of bacterial species and demonstrate the principle of catalase activity and can identify the presence of the aerobic and anaerobic bacteria.

OXIDASE TEST

TEST TUBE METHOD

V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

OXIDASE TEST

AIM:

To find the ability of the organism to produce the cytochrome oxidase enzyme use of oxidase test.

MATERIALS REQUIRED:

A]	APPARATUS & GLASSWARE	:	Test tubes, Glass rod spreader, pipette tips and micropipettes
B]	CHEMICALS	:	Gaby and Hadley reagents and α-naphthol
C]	BACTERIAL CULTURE	:	Overnight cultured colonies

PRINCIPLE:

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase. In the presence of an organism that contains the cytochrome oxidase enzyme, the reduced colorless reagent becomes an oxidized coloured product.

All bacteria that are oxidase-positive are aerobic and can use oxygen as a terminal electron acceptor in respiration. This does NOT mean that they are strict aerobes. Bacteria that are oxidase-negative may be anaerobic, aerobic, or facultative; the oxidase negative result just means that these organisms do not have the cytochrome c oxidase that oxidizes the test reagent. They may respire using other oxidases in electron transport**.**

PROCEDURE:

TEST TUBE METHOD

1. Grow a fresh culture (18 to 24 hours) of bacteria in 4.5 ml of nutrient broth (or standard media that does not contain a high concentration of sugar).
2. Add 0.2 ml of 1% α-naphthol, then add 0.3 ml of 1% paminodimethylaniline oxalate (Gaby and Hadley reagents).
3. Observe for color changes.

DISC METHOD

1. A drop of sample was added to the Oxidase disc.
2. Then the result was observed.

DISC METHOD

Oxidase –ve **Oxidase +ve**

RESULT:

Oxidase positive **:** A Color change to blue within 15 to 30 seconds was observed.

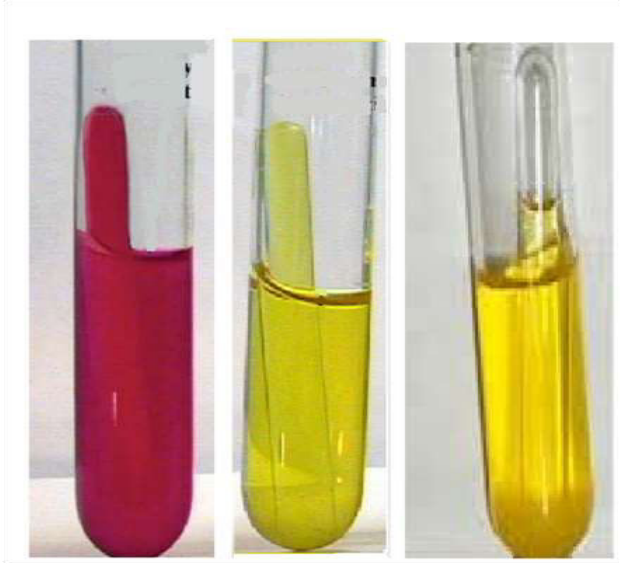
Oxidase negative **:** No change in color was observed.

NUMBER OF BENEFICIARIES: 38

OUTCOME:

Students learnt to screen the suspected colonies of the Enterobacteriaceae family and identify the colonies belonging to the other genera such as *Aeromonas, Pseudomonas, Neisserias, Campylobacter* and *Pasteurella.*

ACID AND GAS PRODUCTION TEST



Carbohydrate fermentation -ve

Carbohydrate fermentation +ve and gas production -ve

Carbohydrate fermentation +ve and gas production +ve

V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

ACID AND GAS PRODUCTION TEST

AIM:

To study the presence of acid and/or gas produced from carbohydrate fermentation.

MATERIALS REQUIRED:

A]	**APPARATUS & GLASSWARE**		Test tubes, Glass rod spreader, pipette tips, micropipettes Bunsen burner, Inoculating loop, Test tubes and Durham tube.
B]	**CHEMICALS**	:	Phenol Red Carbohydrate **broth**
C]	**BACTERIAL CULTURE**	:	24 to 48 hours culture

PRINCIPLE

A fermentation medium is composed of a basal medium containing a specific carbohydrate (glucose, sucrose, or cellulose) along with a pH indicator (phenol red, Andrade's indicator, or bromocresol). When the organism ferments carbohydrates, organic acid products (Lactic acid, formic acid, or acetic acid) are obtained which turns the medium into yellow color with a reduction in the pH (acidic-below pH of 6.8). The change in the pH indicator of the fermentation tube and the gas production in the Durham tube is an indicative of the metabolic reaction with the production of acid as an end product and gas. When microorganisms ferment carbohydrate an acid or acid with gas are produced. Depending upon the organisms involved and the substrate being fermented, the end products may vary. The production of the acid lowers the pH of the test medium, which is detected by the colour change of the pH indicator. Colour change only occurs when sufficient amount of acid is produced, as bacteria may utilize the peptone producing alkaline by-products.

PROCEDURE

A. Preparation of the media

1. Prepare Phenol Red Carbohydrate broth media in 1000 ml of distilled/deionized water and heating gently to dissolve it.
2. Fill 13 x 100 mm test tubes with 4-5 ml of phenol red carbohydrate broth.
3. Insert a Durham tube to detect gas production.
4. Autoclave the prepared test media (at 121°C for 15 minutes) to sterilize. The sterilization process will also drive the broth into the inverted Durham tube.
5. The prepared broth media will be a light red color and the final pH should be 7.4 ± 0.2.

B. Inoculation and Incubation

6. Aseptically inoculate each test tube with the test microorganism using an inoculating needle or loop.
7. Incubate tubes at 35-37°C for 18-24 hours. Longer incubation periods may be required to confirm a negative result.

RESULT

Positive: The development of yellow color in the medium is indicating that positive carbohydrate fermentation reaction.

Negative: Lack of yellow color development is indicating negative carbohydrate fermentation reaction.

Positive: Yellow color development and appearance of gas formation (gas bubbles) in the Durham tube is indicating that positive carbohydrate fermentation reaction and gas production.

NUMBER OF BENEFICIARIES: 38

OUTCOME:

Students learnt to characterize the bacteria that does not produce gas which is used for the fermentation i.e. anaerogenic organisms. They also could be able to determine the fermentation reactions of microorganisms especially Enteric *Bacilli* and *Enterococcus.*

INSTRUMENT - AUTOCLAV E

INSTRUMENT –AUTOCLAVE

Autoclave is also known as steam sterilizer, and is typically used for healthcare or industrial applications. An autoclave is a machine that uses steam under pressure to kill harmful bacteria, viruses, fungi, and spores on items that are placed inside a pressure vessel. The items are heated to an appropriate sterilization temperature for a given amount of time.

Components:

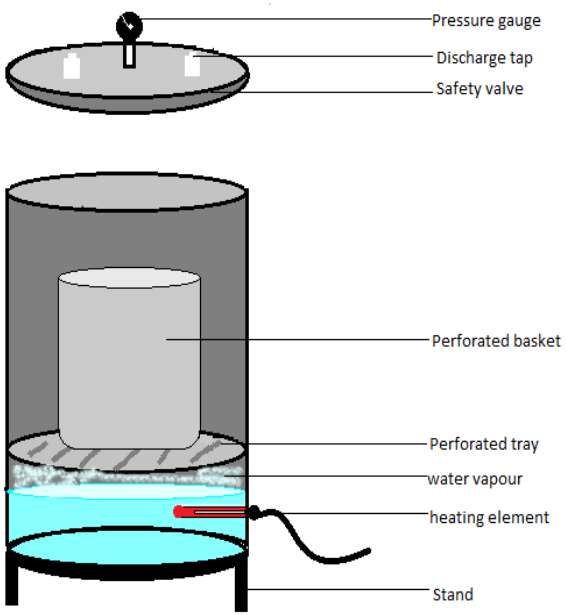
1. Pressure Chamber
2. Lid/ Door
3. Pressure Gauge
4. Pressure releasing Unit
5. Electrical Heater

PROCEDURE:

An autoclave works on a basic principle. The material is directly exposed to steam at the required temperature and pressure for a specific time. The boiling point of water will increase with the rising pressure as a result high temperatures will be generated for sterilization. High-pressure helps in the rapid penetration of heat within the material and sterilization

- Before running an autoclave, check if an item is left inside the autoclave from the previous batch.
- Then check the water level in the autoclave. If the coil is visible then add sufficient water to it and make the coil invisible.
- Now place the material within the autoclave which is needed to sterilize.
- Then close the lid and make it airtight by tightening the screws. After that switched on the autoclave.
- To maintain the required pressure adjust the safety valves.
- Then close the drainage pipe and allow the steam to reach the desired levels (15 lbs).
- After reaching the desired level of pressure, the whistle will blow to remove excess pressure from the autoclave chamber.
- After the whistle, hold the autoclave for 15 minutes, this is known as the holding period.
- Switch off the autoclave and allow it to cool down until the pressure gauge shows that the inside pressure of the autoclave has lowered down to that of the atmospheric pressure.
- The autoclave should not be overcrowded, and the materials should be loaded in a way that ensures sufficient penetration of articles by the steam.
- The items to be autoclaved should always be placed in a secondary container.
- Autoclavable bags are to be used to autoclave packaged waste

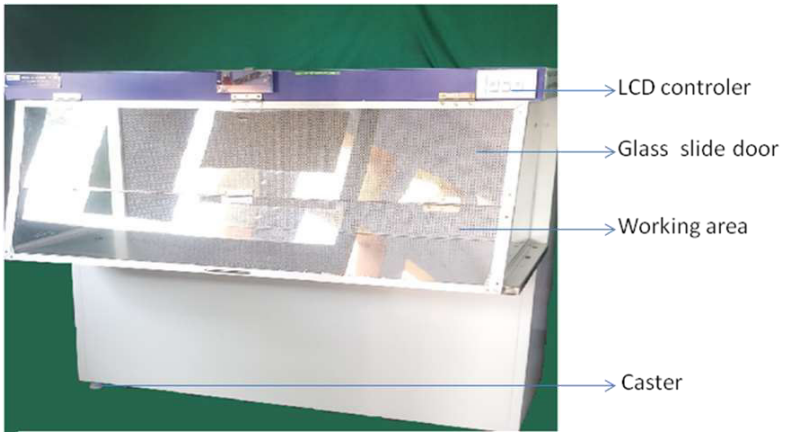
AUTOCLAVE



MAINTENANCE:

- Autoclaves should not be used to sterilize water-proof or water-resistant substances like oil or powders.
- To ensure sufficient penetration, articles should be wrapped in something that allows penetration by steam, and materials like aluminium foils should not be used.
- The items placed inside the chamber should not touch the sides or top of the chamber.
- The wastes and clean items should be autoclaved separately.
- Never attempt to open the lid when the autoclave is working.
- Liquid components should never be autoclaved in sealed containers.
- The liquid inside the containers should only be filled 2/3rd of the total volume to prevent the spilling of the liquid.
- Plastic or polyethylene trays or containers should not be used as they might melt and damage the autoclave.
- Besides, never autoclave flammable, reactive, corrosive, toxic or radioactive materials, household bleach, or paraffin-embedded tissue.
- The paper should not be placed directly inside an autoclave as it is a combustible substance. It should be autoclaved in a waste bag on a bio bag setting to prevent fire.

INSTRUMENT –LAMINAR AIR FLOW CHAMBER



V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

INSTRUMENT –LAMINAR AIR FLOW CHAMBER

A laminar flow cabinet is a carefully enclosed bench designed to prevent contamination of semiconductor wafers, biological samples, or any particle sensitive device. Air is drawn through a HEPA filter and blown in a very smooth, laminar flow towards the user.

WORKING MECHANISM

- Before running the laminar flow cabinet, the cabinet should be checked to ensure that no object susceptible to UV rays is present inside the cabinet.
- The glass shield of the hood is then closed, and the UV light is switched on. The UV light should be kept for about 15 minutes to ensure the surface sterilization of the working bench.
- The UV light is then switched off, and a time period of around 10 minutes is spared before the airflow is switched on.
- About 5 minutes before the operation begins, the airflow is switched on.
- The glass shield is then opened, and the fluorescent light is also switched on during the operation.
- To ensure more protection, the working bench of the cabinet can be sterilized with other disinfectants like 70% alcohol.
- Once the work is completed, the airflow and florescent lamp both are closed and the glass shield is also closed.

PRECAUTIONS

- The laminar flow cabinet should be sterilized with the UV light before and after the operation.
- The UV light and airflow should not be used at the same time.
- No operations should be carried out when the UV light is switched on.
- The person who uses the chamber should be dressed in lab coats and long gloves.
- The working bench, glass shield, and other components present inside the cabinet should be sterilized before and after the completion of work.
- decontamination of the cabinet should be done by wiping down with disinfectant prior to commencing work is mandatory.
- Germicidal UV lamp should be run for 15- 20 minutes prior to using the bench
- Cabinets with UV lights must be turned off during the day when personnel occupy the room.

- Only essential items should be placed in the working place.
- Objects should not be placed between the HEPEX and any point where the clean environment must be maintained.
- We should use proper attire, lab coat, mask, and gloves.

MAINTENANCE

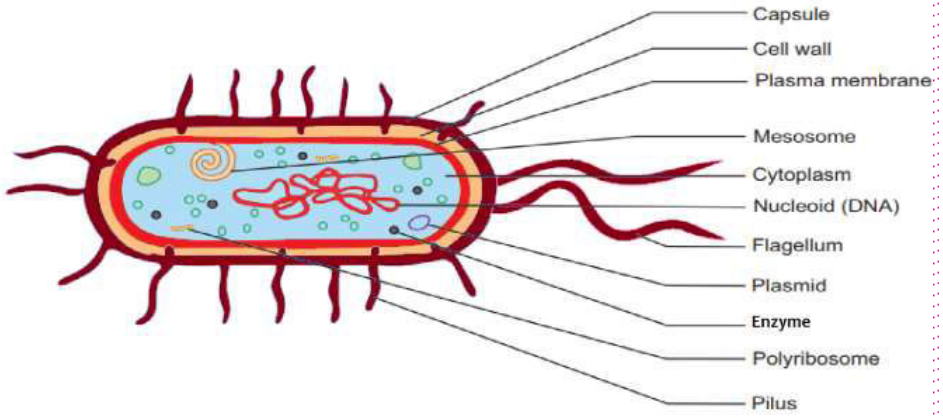
- We should inspect condition of unit and electrical cord/plug to ensure safe operation.
- We should keep the Unit clean. We should wipe down the chamber with a cloth and Sporicidin (We should do Not Use Alcohol or Organic Solvents inside the Unit).
- Pre-filter replacement interval depends on time of use, amount of contaminants and their size. A typical period is every 3 months.
- The HEPA filter should be replaced when the efflux velocity cannot be maintained at 90 LFPM.
- Annual schedule certification and routine maintenance with vendor is mandatory.

V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

MICROBIAL TAXONOMY

[OBSERVATION OF PERMANENT SLIDES]

E. COLI

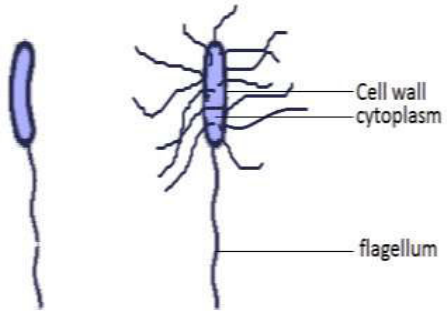


V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

E. COLI

- *Escherichia coli* is a Gram-negative, facultative anaerobic rod-shaped bacterium.
- It is about 2.0µm long and 0.25–1.0 µm in diameter, with a cell volume of 0.6–0.7 μm^3.
- It is s commonly found in the lower intestine of warm-blooded organisms.
- Strains that possess flagella are motile. The flagella have a peritrichous arrangement. It also attaches and effaces to the microvilli of the intestines
- The envelope of *E. coli* has three layers: cytoplasmic membrane, peptidoglycan, and outer membrane.
- Its cell wall consists of an outer membrane containing lipopolysaccharides, a periplasmic space with a peptidoglycan layer, and the inner membrane is cytoplasmic membrane.
- The chromosome or nucleoid is composed of the genomic DNA, RNA, and protein.
- The harmless strains are part of the normal microbiota of the gut, and can benefit their hosts by producing vitamin K2 which helps blood to clot and preventing colonisation of the intestine with pathogenic bacteria having a mutualistic relationship.
- *E. coli* is a preferred host for gene cloning due to the high efficiency of introduction of DNA molecules into cells. It is a preferred host for protein production due to its rapid growth and the ability to express proteins at very high levels.

VIBRIO CHOLERA

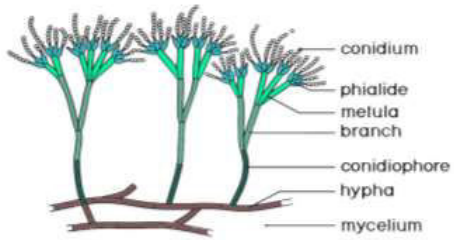


V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

VIBRIO CHOLERA

1. *Vibrio cholerae* is a species of Gram-negative, motile, facultative anaerobic bacteria and comma-shaped bacteria.
2. The bacteria naturally live in brackish or saltwater where they attach themselves easily to the chitin-containing shells of crabs, shrimps, and other shellfish. It is typically found in salt water,
3. The bacterium has a flagellum at one pole and several pili throughout its cell surface.
4. Initial isolates are slightly curved, whereas they can appear as straight rods upon laboratory culturing.
5. Its motility depends on a single polar flagellum. The bacterium has a flagellum at one cell pole and several pili throughout its cell surface.
6. It tolerates alkaline media that kill most intestinal commensals, but they are sensitive to acid.
7. It measures 0.3 μm in diameter and 1.3 μm in length with average swimming velocity of around 75.4μm/sec.
8. *Vibrio cholera* cell wall composed of Lipopolysaccharides and the Chemical analysis revealed the presence of glucose, fructose, mannose, heptose, rhamnose, ethanolamine, fatty acids and glucosamine.
9. *V. cholerae* infect the intestine and it produces a toxin that causes the lethal diarrhea of cholera.
10. Infection can be spread by eating contaminated food or drinking contaminated water.
11. Vibrio species are facultative anaerobes that respond positive for oxidase test.

PENICILLIUM

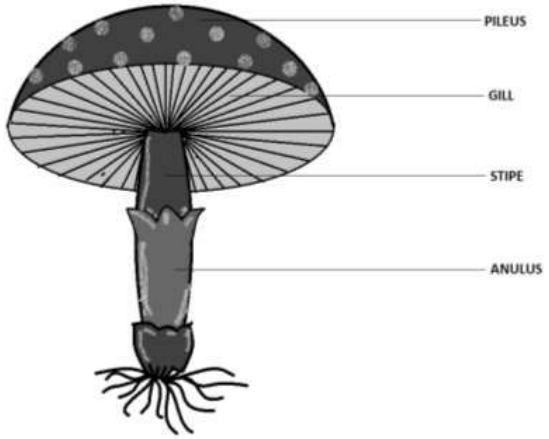


V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

PENICILLIUM

- Penicillium is a genus of saprophytic (feeding on dead and decaying materials) fungi.
- They are commonly known as blue or green mould. The vegetative structure of Penicillium is a multicellular mycelium
- The mycelium is made up of highly branched, multinucleated and septate long thread-like filamentous structure known as hyphae
- The cell wall is made up of a glucose polysaccharide and chitin
- The cytoplasmic continuity is maintained through central pore, present in the septa
- Conidiophores are present at the branch ending along with spherical conidiospores, which are asexual spores produced exogenously
- Conidia are produced in the basipetal succession, i.e. the youngest conidium is present at the base.
- Conidia are produced from the specialized cells called phialide, present in the group and give a brush-like appearance to the fungus
- Ascospores are found in asci arranged in ascocarps, which are sexual spores produced endogenously
- The food is stored in the form of oil globules.
- They are economically important for the production of cheese, organic acids and antibiotics.
- They also play an important role as a decomposer in the ecosystem.

AGARICUS BISPORUS

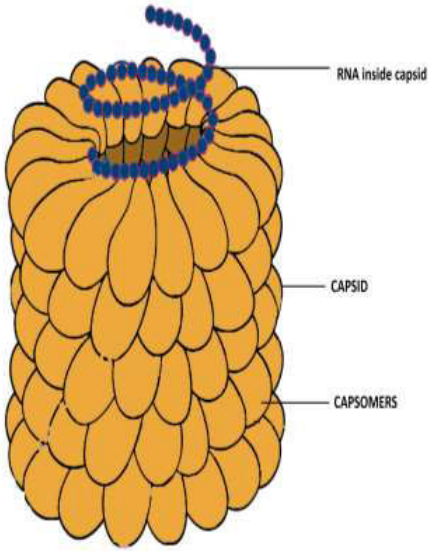


V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

AGARICUS BISPORUS

1. Agaricus is a genus of mushrooms containing both edible and poisonous species over 300 members worldwide.
2. The genus includes the common button mushroom is Agaricus bisporus.
3. *Agaricus bisporus* is an edible basidiomycete mushroom, native to grasslands in Europe and North America.
4. It is found nearly always in grassland.
5. It has two colour states while immature – white and brown in mature state
6. Members of *Agaricus* are characterized by having a fleshy cap or pileus, from the underside of which grow a number of radiating plates or gills on which are produced the naked spores.
7. They are distinguished from other members of their family, Agaricaceae.
8. Members of *Agaricus* also have a stem or stipe which elevates it above the object on which the mushroom grows and a partial veil, which protects the developing gills and later forms a ring or annulus on the stalk.
9. Agaricus is an edible fungus, cosmopolitan in distribution and is commonly known as mushroom
10. This saprophytic fungus is seen during rainy season found growing on soil humus, decaying litter on forest floors, in the fields and lawns, wood logs and manure piles.
11. Agaricus is used for cancer, type 2 diabetes, high cholesterol, hardening of the arteries, liver disease, bloodstream disorders, and digestive problems.
12. Other uses include prevention of heart disease, weakened bones (osteoporosis), and stomach ulcers

VIRUS- TMV

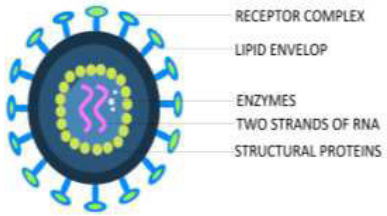


V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

VIRUS- TMV

1. Tobacco Mosaic Virus (TMV) is a positive-sense single-stranded RNA virus species in the genus Tobamo virus that infects a wide range of plants.
2. The infection causes characteristic patterns, such as mosaic-like mottling and discoloration on the leaves.
3. This virus has a rod-like appearance.
4. Its capsid is made from 2130 molecules of coat protein and one molecule of genomic single strand RNA, 6400 bases long.
5. The coat protein self-assembles into the rod-like helical structure (16.3 proteins per helix turn) around the RNA, which forms a hairpin loop structure.
6. The TMV genome consists of a 6.3–6.5 kbp single-stranded (ss) RNA.
7. The protein monomer consists of 158 amino acids which are assembled into four main alpha-helices, which are joined by a prominent loop proximal to the axis of the virion.
8. It is the first pathogen identified as a virus. It was crystallized by W.M.Stanley.
9. TMV is spread mechanically by abrasion with infected sap.
10. Symptoms of virus infection include colour changes, dwarfing, and tissue distortion.
11. The appearance of streaks of colour in certain tulips is caused by virus.

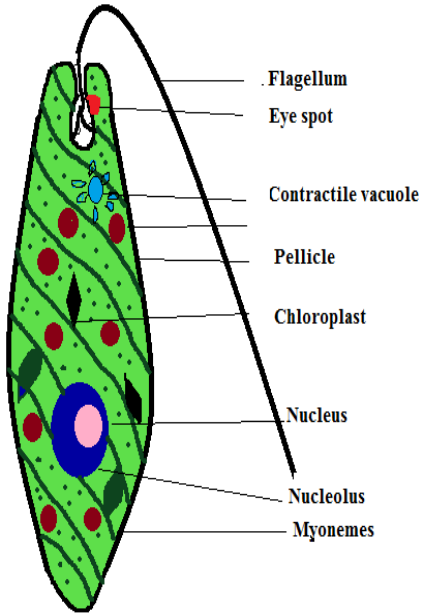
HIV



HIV

- HIV (Human Immunodeficiency Virus) is a virus that attacks the body's immune system.
- It can lead to AIDS (acquired immunodeficiency syndrome).
- HIV is different in structure from other retroviruses.
- The mature HIV particle (virion) is round, measures approximately 100 nm in diameter.
- It consists with an outer lipid membrane as its envelope.
- Its innermost region consists of a cone-shaped core that includes two copies of the (positive sense) ssRNA genome, the enzymes reverse transcriptase, integrase and protease, some minor proteins, and the major core protein.
- The genome of HIV, which is composed of two strands of RNA.
- It is packaged inside a distinctive cone-shaped capsid, which protects the RNA and delivers it to the cells that HIV infects.
- The virus can be transmitted through contact with infected blood, semen or vaginal fluids.
- Within a few weeks of HIV infection, flu-like symptoms such as fever, sore throat and fatigue can occur.
- The disease is usually asymptomatic until it progresses to AIDS. AIDS symptoms include weight loss, fever or night sweats, fatigue and recurrent infections.
- No cure exists for AIDS, but strict adherence to antiretroviral regimens (ARVs) can dramatically slow down the disease's progress as well as prevents secondary infections and complications.

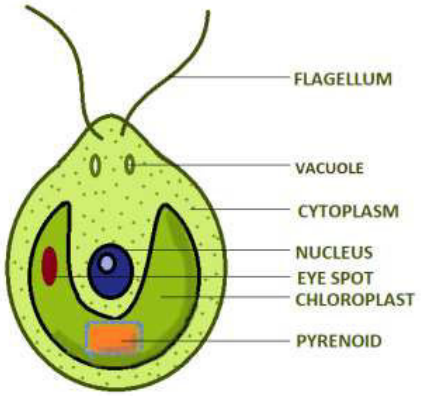
PROTOZOAN - EUGLENA



PROTOZOAN- EUGLENA

- *Euglena* is a green coloured single cell eukaryotic flagellate protozoan, inhabits on the surface of freshwater.
- It is an intermediate link between Plant Kingdom and Animal Kingdom.
- It is unicellular and hence placed in the Phylum Protozoa.
- It moves with the help of flagella and so included in the class Flagellata.
- Numerous chloroplasts are arranged in star-shaped.
- It is spindle shaped.
- It is covered by a pellicle.
- It has a single nucleus.
- It absorbs dissolved oxygen from the surrounding water by diffusion.
- Both holophytic and saprozoic modes of nutrition are seen.
- The contractile vacuole discharges the excess of water.
- An eye spot helps in photosensitivity.
- Euglena produces two daughter individuals by longitudinal binary fission.
- Species of *Euglena* are found in fresh water and salt water.
- They are often abundant in quiet inland waters where they may bloom in numbers sufficient to color the surface of ponds and ditches green or red.

CHLAMYDOMONAS



V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

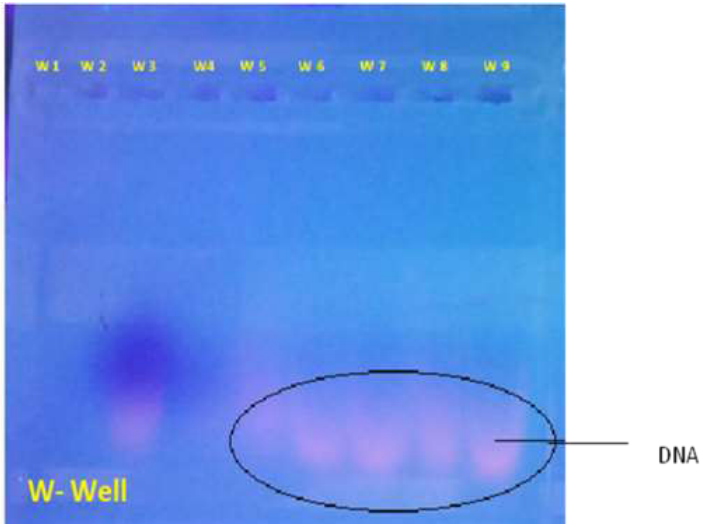
CHLAMYDOMONAS

- Chlamydomonas is a pear-shaped, biflagellated single-celled green algae.
- It is found in stagnant water of freshwater, seawater and on damp soil, and even in snow as "snow algae".
- Cell wall is made up of a glycoprotein and non-cellulosic polysaccharides instead of cellulose.
- Two anteriorly inserted whiplash flagella are present. Each flagellum originates from a basal granule in the anterior papillate or non-papillate region of the cytoplasm.
- Contractile vacuoles are near the bases of flagella.
- Prominent cup or bowl-shaped chloroplast is present.
- The chloroplast contains bands composed of a variable number of the photosynthetic thylakoids which are not organised into grana-like structures.
- The nucleus is enclosed in a cup-shaped chloroplast, which has a single large pyrenoid where starch is formed from photosynthetic products.
- Pyrenoid with starch sheath is present in the posterior end of the chloroplast.
- Eye spot present in the anterior portion of the chloroplast. It consists of two or three, more or less parallel rows of linearly arranged fat droplets.
- Asexual reproduction occurs by zoospores, aplanospores and hypnospores and Sexual reproduction is through isogamy, anisogamy or oogamy.

V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

BIOTECHNOLOGY

ISOLATION OF CHROMOSOMAL DNA FROM MICROBES



V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

ISOLATION OF CHROMOSOMAL DNA FROM MICROBES

AIM: To isolate chromosomal DNA from bacteria.

MATERIALS REQUIRED:

A] EQUIPMENT	**:**	Shaker, Laminar Air flow chamber, Microcentrifuge and Incubatorand Agarose gel electrophoresis
B] GLASSWARE AND APPARATUS:		Sterile 1.5 ml tubes, Beaker, 2 ml Eppendorf tubes, Micropipettes, and pipette tips
C] CHEMICALS	:	Lysis buffer, Denaturing solution, Potassium acetate (pH 4.8), Sodium acetate (pH 5.2), 0.2N Sodium hydroxide, SDS, Ethyl alcohol and TE buffer,
D] CULTURE	**:**	Overnight bacterial culture.

PRINCIPLE:

Extraction and purification of plasmid DNA is very useful technique in modern molecular biology studies. Plasmid is performed in 3 stages. In stage 1 bacterial cell wall is weakened and lysed by EDTA and a detergent at high pH. In stage 2, insoluble cell debris consisting of genomic DNA and protein is precipitated with high salt content. In stage 3, plasmid DNA is precipitated in the presence of ethyl alcohol.

PROCEDURE:

- Take 2 ml of bacterial culture (Ex. *E.coli* culture) in two sterile eppendorf tubes.
- Centrifuge at 5000 rpm for 7- 10 minutes at 4^{o}C.
- Decant the culture supernatant.
- Resuspend the cell pellet in 100µl of ice cold lysis buffer (pH 8.0)
- Mix by vortexing and keep at RT for 5minutes.
- Add 200µl of freshly prepared alkaline solution containing 0.2N NaOH and 2% SDS.
- Mix gently by inverting the tube rapidly two or three times and incubate the tubes on ice for 5 minutes.
- Add 150µl of ice cold potassium acetate and mix thoroughly by inverting the tube.
- Store on ice for 10 minutes.

- Centrifuge at 10,000 rpm for 5 minutes at 4°C.
- Transfer the supernatant carefully to fresh eppendorf and add 2 volumes of ice cold ethyl alcohol.
- Incubate the tubes at 0°C for 30 minutes and centrifuge at 10,000rpm for 10 minutes at 4°C.
- Discard the supernatant and wash the DNA pellet with 70% ethyl alcohol.
- Air dry the pellet and dissolve in 100ul of TE buffer.
- Electrophoreses can performed with 5µl in 0.7% agarose gel to estimate yield of DNA.

RESULT:

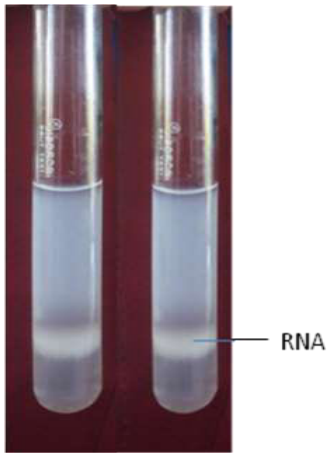
Chromosomal DNA was observed and it was confirmed with Agarose gel electrophoresis.

NUMBER OF BENEFICIARIES: 38

OUTCOME:

Students gained practical skill on culture of microorganism and preparation of chromosomal DNA from microbes.

ISOLATION OF RNA FROM SPLEEN/LIVER



ISOLATION OF RNA FROM SPLEEN/LIVER

AIM

To isolate total RNA from liver/ spleen.

MATERIALS REQUIRED:

A] EQUIPMENT	:	Centrifuge and Homogenizer
B] GLASSWARE AND APPARATUS:	:	Glass rod, Centrifuge tube (plastic), Conical flask, Beaker, Test tubes and Pipettes.
C] CHEMICALS	:	80% phenol, Acetate Buffer –0.05M (pH 5 .1), SDS, Diethyl Pyrocarbonate-0.05%, Chloroform, Isoamyl Alcohol, Ethyl Alcohol, NaCl-0.17M
D] CULTURE	:	Liver/Spleen,

PRINCIPLE:

RNA refers to Ribonucleic acid which is the product encoded by a process is called transcription of DNA, the genetic information storer. Different types of RNAs (mRNA, tRNA, rRNA, SnRNA) are exists in the cell. On translation, the RNA gives protein. All the steps should be carried out at 0-4º C unless mentioned in the protocol .All the glasswares and buffers should be sterilized.

PROCEDURE

- Cut the liver into small pieces and wash with saline (0.15M NaCl).
- Homogenize in 0.05M acetate buffer at pH 5.1 in the presence of 0.05% diethyl Pyrocarbonate.
- Add SDS to a final concentration of 1% and mix.
- Add equal volume of hot 80% phenol (65ºC).
- Mix thoroughly and equilibrate at 65°C for 15 min.
- Centrifuge at 3000 rpm for 15 minutes. Remove the aqueous phase using a pipette and save.
- Add equal volume of 80% phenol and equal volume of chloroform: isoamyl alcohol (24:1).
- Shake thoroughly and centrifuge at 3000 rpm for 15 minutes.
- Remove the aqueous phase with a pipette.

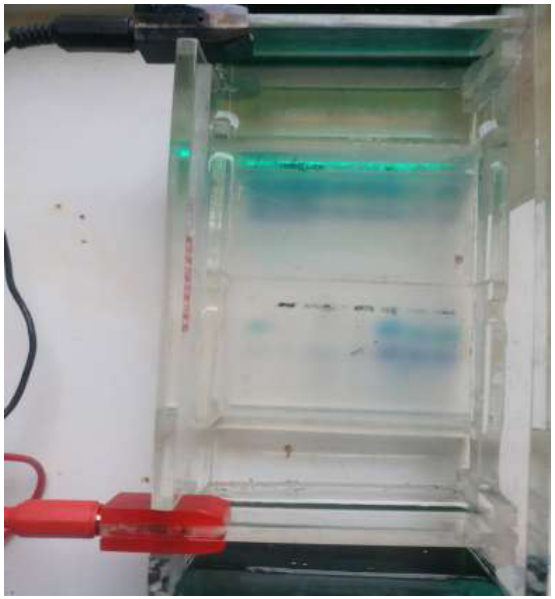
- Pool aqueous phases and add 2.5 volume of ethyl alcohol in the presence of 0.17M NaCl. Mix and store at -20°C.

RESULT : RNA strands are obtained as a white mass or turbidity.

NUMBER OF BENEFICIARIES: 38

OUTCOME: Students learnt the technique on isolation of RNA from animal tissues using Spleen/Liver.

SEPARATION OF DNA BY AGAROSE GEL ELECTROPHORESIS



V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

SEPARATION OF DNA BY AGAROSE GEL ELECTROPHORESIS

AIM:

To check the presence of the isolated DNA by performing Agarose gel electrophoresis.

MATERIALS REQUIRED:

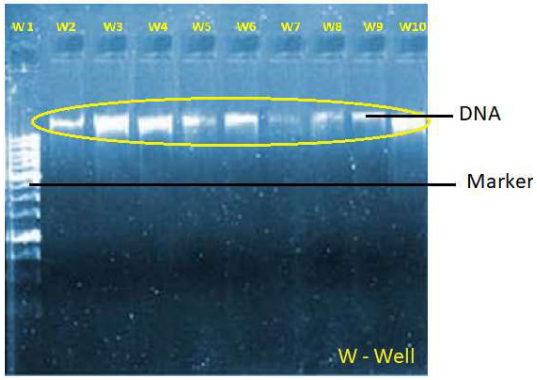
A] GLASSWARE AND APPARATUS:	Agarose Gel Electrophoresis Apparatus, Micropipette.
B] CHEMICALS	Beaker, Conical flask, Test tubes, Eppendorf tubes and Microtips..
C] CULTURE	TAE buffer, Ethidium bromide and Loading Dye (Contains Glycerol and Bromophenol blue).

PRINCIPLE:

Electrophoresis refers to the separation of macromolecules of different size by application of a constant electric field on to the DNA fragments placed in a matrix of polymerized agarose. As DNA molecule is negatively charged and travels towards the anode, it is loaded at the cathode end. The speed of migration of the fragments has an inverse relation with the size of DNA. The separated fragments are visualized by staining the gels with an intercalating dye (ethidium bromide), which fluoresces under UV light. Acrylamide gel is used for separation of small fragments of DNA (5 to 500bp). Agarose gels can resolve DNA fragments varying in size from 200bp to about 50kb depending upon the concentration of agarose in the gel.

PROCEDURE:

1. Seal the open ends of a clean gel platform using an adhesive tape so as to form a mould and place it on an even horizontal surface.
2. Place the comb at about one cm from one end of the platform, at a height of about one millimeter from the surface of the plate.
3. Measure out 30 ml of 1X TAE electrophoresis buffer in a conical flask and add 300mg of agarose to give 1% concentration. Dissolve the agarose and melt in the flask by keeping it in a boiling water bath.
4. Cool the melted agarose to about 55 to 60ºC and add 0.1µl ethidium bromide.
5. Then pour the agarose solution slowly into the gel platform, without the inclusion of air bubble.



Sample Running in Agarose Gel Electrophoresis

V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

6. Allow the gel to set at room temperature for about 30 minutes and carefully remove the comb and adhesive tap, without disturbing or damaging the slots/wells.
7. Place the gel along with the platform into electrophoresis chamber/tank and pour the TAE buffer into the chamber to a height of about 1mm above the gel.
8. Mix the DNA samples (5-10µl) with a loading dye and slowly load the mixtures into the slots/wells from left to right using a micropipette. Adequate care should be taken to avoid air bubbles and piercing of the gel.
9. Add standard DNA marker (1µg) at the last slot/well.
10. Place the lid and give the power supply. In the beginning, a pulse current of 10 V/cm.
11. Stop the electrophoresis as soon as the bromophenol blue (tracking dye) reaches the appropriate place (ie. 75% of the gel size).
12. Visualize DNA bands under UV transilluminator.

RESULT: The isolated DNA was observed and confirmed using Agarose gel electrophoresis.

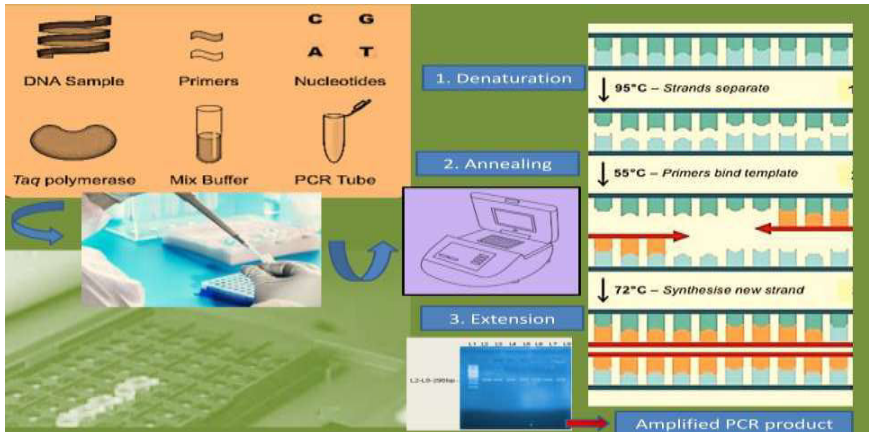
NUMBER OF BENEFICIARIES: 38

OUTCOME:

Students acquired technical skill on working mechanism of Agarose gel electrophoresis and they could be able to handle the apparatus individually.

POLYMERASE CHAIN REACTION (PCR) - DEMONSTRATION

STEPS IN PCR



PCR MIXTURE (50µL REACTION MIX)

Chemicals	Stock concentration	Reaction concentration	50µl reaction mix
Taq buffer	10X	1X	5.0 µl
$MgCl_2$	25mM	1.0mM	2.0 µl
dNTP's	10mM	200µM	1.0 µl
Forward primer	10µM	0.6µM	3.0 µl
Reverse primer	10µM	0.6µM	3.0 µl
dH_2O			31.0 µl
Taq polymerase	5U	2.5U	0.5 µl
Template		50ng	2.5µl
DMSO		2%	2.0µl

PCR CONDITION USED

Steps	Temperature	Time	Cycle
Initial denaturation	94ºC	5 min	
Denaturation	94ºC	30 sec	
Annealing	61ºC	30 sec	35 cycles
Extension	72ºC	30 sec	
Final extension	72ºC	7 min	

V.V.Vanniaperumal College for Women, Virudhunagar Tamilnadu.
No HRD-11011/163/2020-HRD-DBT-Zoology/Lab Manual – Microbiology & Biotechnology

POLYMERASE CHAIN REACTION (PCR) - DEMONSTRATION

AIM: To study the working mechanism and applications of PCR.

MATERIALS REQUIRED

A] EQUIPMENTS	: PCR, Magnetic stirrer
B]GLASSWARE AND APPARATUS	: Micro tubes, Micropipettes tips, Beaker, Conical flask and test tubes.
C] CHEMICALS	: Buffer, all four deoxyribonucleoside triphosphates, Primers, Taq polymerase, co-factors of enzyme, DNA sample and water.

PRINCIPLE:

The PCR technique is based on the enzymatic replication of DNA. In PCR, a short segment of DNA is amplified using primer mediated enzymes. DNA Polymerase synthesizes new strands of DNA complementary to the template DNA. The DNA polymerase can add a nucleotide to the pre-existing 3'-OH group only. Therefore, a primer is required. Thus, more nucleotides are added to the 3' prime end of the DNA polymerase.

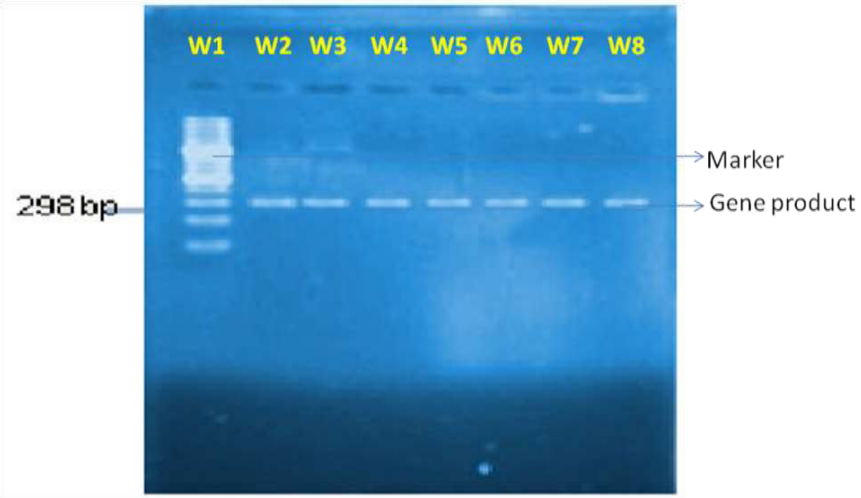
PROCEDURE:

Typically, PCR consists of a series of 20–40 repeated temperature changes, called thermal cycling.

INITIALIZATION: This step is required for DNA polymerases that require heat activation only by hot start PCR. It requires heating the reaction chamber to a temperature of 94–96 °C (201–205 °F) for 1–10 minutes.

DENATURATION: This step is the first regular cycling event and heating the reaction chamber to 94–98 °C (201–208 °F) for 20–30 seconds. This causes denaturation of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.

PCR PRODUCTS



ANNEALING: In this step, the reaction temperature is lowered to 50–65 °C (122–149 °F) for 20–40 seconds, allowing annealing of the primers to each of the single-stranded DNA templates. Two different primers are typically included in the reaction mixture: one for each of the two single-stranded complements containing the target region. The primers are single-stranded sequences themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3' end of each strand.

EXTENSION/ELONGATION: The temperature at this step depends on the DNA polymerase used, the optimum activity temperature for the thermostable DNA polymerase of Taq polymerase is approximately 75–80 °C (167–176 °F). The temperature, 72 °C (162 °F) is commonly used with this enzyme. In this step, the DNA polymerase synthesizes a new DNA strand which is complementary to the DNA template strand by adding free dNTPs from the reaction mixture that is complementary to the template in the 5'-to-3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (elongating) DNA strand.

The processes of denaturation, annealing and elongation constitute a single cycle. Multiple cycles are required to amplify the DNA target to millions of copies.

FINAL ELONGATION: This single step is optional, but is performed at a temperature of 70–74 °C (158–165 °F) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.

FINAL HOLD : The final step cools the reaction chamber to 4–15 °C (39–59 °F) for an indefinite time and may be employed for short-term storage of the PCR products.

RESULT:

PCR amplified gene product was observed.

NUMBER OF BENEFICIARIES: 38

OUTCOME:

Students gained knowledge on the technique in handling of PCR and its applications.

MS MEDIA COMPOSITION AND PREPARATION

Stock solution of Macro-salts (20X)

Constituents	Amount (gm) to be taken for stock solution (20X)	Final volume of stock (ml)
$(NH_4)NO_3$	33.0	1000
KNO_3	38.0	
$CaCl_2.2H_2O$	8.8	
KH_2PO_4	3.4	
$MgSO_4.7H_2O$	7.4	

To make 1 litre of macro-salt stock solution, dissolve the salts one after the other in 800 ml of DDH_2O and then make it upto 1000ml. The solution is filtered and stored in refrigerator (10-16^oC) for long period.

STOCK SOLUTION OF MICRO-SALTS (100X)		
Constituents	Amount (mg) to be taken for stock solution (100X)	Final volume of stock (ml)
H_3BO_3	620	100
$NA_2MoO_4.2H_2O$	25	
$CoCl_2.6H_2O$	2.5	
$CuSO_4.5H_2O$	2.5	
$ZnSO_4.7H_2O$	860	
$MnSO_4.4H_20$	2230	
KI	83	

STOCK SOLUTION OF MS THREE VITAMINS (1000 X)				
Constituent	Amount (50X) to be taken for stock solution in mg.	Final volume (ml)	Storage temperature (^oC)	Duration of storage (in days)
Thiamine HCl	5	50	0	15
Nicotinic acid	25			
Pyridoxine HCl	25			
Glycine	100			

MS MEDIA COMPOSITION AND PREPARATION

AIM: To prepare Murashige-Skoog culture medium for culturing the microorganisms.

MATERIAL REQUIRED:

A] EQUIPMENTS	:	PCR, Magnetic stirrer
B] GLASSWARE AND APPARATUS	:	Clinical flask, Pipettes, Beaker, Screw capped bottles, Measuring cylinder Reagent bottle, Buchner funnel, filter paper and suction pump
C] CHEMICALS	:	Activated charcoal, Ammonium nitrate, Boric acid, Calcium chloride- dehydrate, Cane sugar, Cobalt chloride- hexahydrate, Copper sulphate- heptahydrate, Disodium EDTA, Disodium molybdate, Ethyl alcohol, Ferrous sulphate- heptahydrate, Glycine, Indole acetic acid (IAA), Magnesium chloride- heptahydrate, Manganese sulfate-tetrahydrate, Meso- inositol, Nicotinic acid,Potassium dihydrogen phosphate, Potassium iodide, Potassium nitrate, Pyridoxine HCl, Thiamine HCl, Zinc sulfate- heptahydrate.

PREPARATION OF STOCK SOLUTIONS:

Stock solution of Iron (200X):

Dissolve 745mg of Na_2EDTA in 75 ml of boiling DDH_2O, then add 557mg of $FeSO4.7H_2O$ gradually. Keep on a magnetic stirrer for 1 hr in hot condition until the colour of the solution changes to golden yellow. Finally make the solution to 100 ml and store in refrigerator (5^oC) in amber coloured bottle.

Stock solution of Meso- inositol (500X):

Dissolve 1 gm Meso- inositol in 20 ml of DDH_2O. Store at 0^oC for 15 days.

Stock solution of Glycine (1000X):

Dissolve 40 mg glycine in 20 ml of DDH_2O. Store at 0^oC for 15 days.

Stock solution of Hormone (10X):

The stock solution of hormones is prepared by dissolving 10 mg of crystals in water miscible solvent and then make up with DDH_2O. Store at 0^oC for 15 days.

PRINCIPLE:

The culture medium refers to medium containing nutrients eg. Inorganic salts, iron source, vitamins, amino acids, growth hormones and carbohydrate sources which enhances the growth of a culture in -vitro under controlled conditions. The most preferred and frequency used carbon source is sucrose and solidifying agent is agar. The medium can be classified as natural or artificial medium defined or undefined medium. The medium is prepared to culture cells/organ/ tissues *in vitro*. The composition of the medium determines the growth rate of the cells/organ/ tissues.

PROCEDURE:

The media is prepared with utmost care so that contamination of the medium is prevented.

To prepare 1 litre of MS medium:

- Dissolve 30 gms of cane sugar in 200 ml of DDH_2O. Mix 1-2 gms of activated charcoal and filter through the filter paper, set inside the Buchner funnel fitted on a conical flask with side arm attachment. Filtering is performed using a suction pump.
- Take DDH_2O in another flask and add in sequence the required amount of stock solutions as follows :-

i)	Stock solution of macrosalts	50ml
ii)	Stock solution of microsalts	1ml
iii)	Stock solution of Fe-EDTA	5ml
iv)	Stock solution of MS 3 Vitamins	1ml
v)	Stock solution of Glycine	1ml
vi)	Stock solution of meso-inositol	2ml

Desired concentration of Auxin/ Cytokinin are added from the stock solution according to the following formula

$$\frac{\text{Desired Concentration}}{\text{Medium}} = \frac{\text{Amount of stock solution to be taken for 1 litre}}{\text{Stock solution}}$$

- Pour the filtered sucrose solution and salt and vitamins, amino acid, hormone solution mixture into 1 litre measuring cylinder and make it upto 1 litre with DDH_2O by uniform mixing.
- Adjust the pH to 5.7 using 0.1N NaOH, using pH meter.
- Add 8 gms of agar to the liquid medium to make solid medium. Melt in hot air oven.
- Dispense the culture medium into culture tube. Close the mouth of the culture tube using non-absorbent cotton plug covered with gauze. Cover it with brown paper and rubber band.
- Medium is sterilized.

RESULT:

Murashige-Skoog culture medium was prepared and used for culturing the microorganisms.

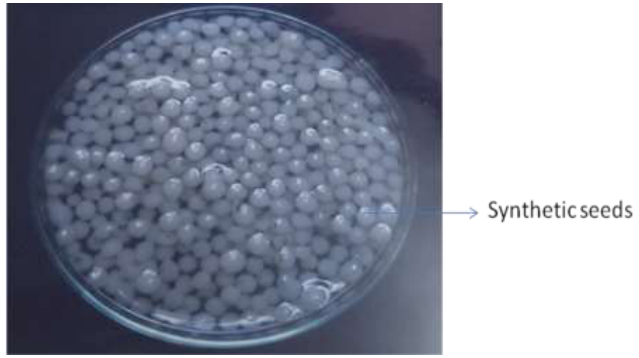
NUMBER OF BENEFICIARIES: 38

OUTCOME:

Students learnt the technique of preparing the Murashige-Skoog culture medium manually in laboratory condition.

SYNTHETIC SEED PREPARATION

SYNTHETIC SEEDS



SYNTHETIC SEED PREPARATION

AIM: To prepare synthetic seeds using chemical method in the laboratory.

MATERIALS REQUIRED:

A] EQUIPMENTS	:	Laminar Air Flow Chamber,
B] GLASSWARE AND APPARATUS	:	Micropipepette, Petriplates, Forceps, Scalpels, Microtips, Pasteur pipette and Beaker.
C] CHEMICALS	:	1. 3% Sodium alginate and 100mM Ca Cl_2. 2. MS liquid medium and Sterile distilled water.
D] BIOLOGICAL SAMPLES	:	Explant: Shoot tips of *Cathranthus roseus*

PRINCIPLE:

Synthetic seeds are living seed like structures made experimentally. Regenerable explants derived from tissue culture are encapsulated in an artificial polymer matrix. The matrix acts as an artificial seed coat and provides sufficient nutrients and developmental control agents. They behave like true seeds and generate complete plants. Entrapment of explants within spheres of calcium alginate gel has now become the most widely used technique for immobilizing living cells. Advantages of synthetic seed technology include economy of space, easy transport, storage etc.

PROCEDURE:

- Excise shoot tip is taken from in vitro micro propagated *Cathranthus roseus* plant.
- Prepare 3% Sodium alginate in 100 ml of MS liquid medium with 3% Sucrose.
- Prepare 100mM $CaCl_2$ in sterile distilled water.
- Sterilize both solutions at 120ºC for 18 minutes and allow it to cool.
- Aseptically transfer the explants to sodium alginate solution and incubate at RT for 15-25 minutes.
- Alginate solution along with explants using a sterile Pasteur pipette drop into Calcium chloride solution.
- Allow it to remain under light for 30 minutes.
- Decant $CaCl_2$ and wash the beads in MS liquid medium minus hormones for hormone concentration.
- Dry the seeds and store it for future culturing work.

RESULT:

The synthetic seeds were prepared from Shoot tips of *Cathranthus roseus* by adopting chemical method using Sodium alginate and Calcium chloride

NUMBER OF BENEFICIARIES: 38

OUTCOME: Students learnt the technique of synthetic seed preparation and gained the knowledge on Synthetic seed preparation under laboratory condition.