KARPAGAM ACADEMY OF HIGHER EDUCATION
(Deemed to be University Established Under Section 3 of UGC Act 1956)
Pollachi Main Road, Eachanari Post, Coimbatore - 641 021. INDIA
Phone : 0422-6471113-5, 6453777 Fax No : 0422 -2980022-3
Email : info@karpagam.com Web : www.kahedu.edu.in

DEPARTMENT OF BIOCHEMISTRY

SUBJECT NAME: PRACTICAL II - PLANT BIOCHEMISTRY AND MICROBIOLOGY

SUB.CODE: 17BCP112

SEMESTER: I **CLASS: I M.Sc., BIOCHEMISTRY**

PLANT BIOCHEMISTRY

1. Phytochemical screening of any one selected medicinal plant
2. Estimation of Tannins
3. Estimation of Flavonoids
4. Estimation of Chlorophyll
5. Estimation of Phenols

MICROBIOLOGY

6. Isolation of pure culture – serial dilution, pour plate, spread plate, streak plate methods.
7. Colony morphology – colony counting.
8. Staining techniques- simple, differential, spore, and fungal staining.
9. Antibiotic resistance / sensitivity test (Disc method)
10. Estimation of bacteria- growth curve of bacteria and generation time.
11. Identification of microorganisms – biochemical tests (IMVIC test)(Group Experiment)
12. Microbiology of potable water
13. Isolation, characterization and purification of ANY one of the following microbial enzymes
    a) Amylase
    b) Protease
14. Assay of Antibacterial of ANY ONE selected medicinal plant by Disc or Well diffusion and broth dilution method.
15. Assay of antifungal activity of ANY ONE selected medicinal plant by Disc or Well diffusion. TLC- Bioautography.

PLANT TISSUE CULTURE (Group experiment)

16. Preparation of tissue culture media
17. Surface sterilization
18. Induction of meristem culture

19. Callus induction.
20. Regeneration of shoot and root from callus culture.

REFERENCES

1. Sadasivam, S.,and Manickam, A., (2009). Biochemical Methods, New Age, International Publishers, New Delhi.
2. Rajan S., and Selvi C., (2011). Experimental Procedures in Life Sciences. Anjana Book House, Chennai.

PREPARATION OF DIFFERENT PLANT EXTRACTS

Preparation of petroleum ether extract:

About 25g of powered plant material was extracted with 125ml petroleum by using a separating funnel with occasional shaking for 16 hours. The extract was concentrated by Rotary flask evaporator (Buchi type)

Each time before extracting with the next solvent the residue was dried thoroughly to remove the solvent used.

Preparation of Chloroform extract:

After complete drying, the above said residue was extracted with 125ml chloroform by occasional shaking for 16 hours.

Preparation of ethyl acetate extract:

The above said residue was extracted with 125ml ethyl acetate by occasional shaking for 16 hours.

Preparation of ethanol extract:

The above said residue was extracted with 125ml ethanol by occasional shaking for 16 hours.

Preparation of water extract:

The above said residue was extracted with 125ml water by occasional shaking for 16 hours. The flow chart for extraction procedure is given in the table.

EX. NO. 1

PHYTOCHEMICAL SCREENING TEST

Phytochemical screening of any one selected medicinal plant

Phytochemical screening is done for analyzing secondary metabolites that are responsible for curing ailments. The Phytochemical screening of the plant extract was carried out by following the method of Trease and Evans (1978) and Harborne (1984).

Test for alkaloids:

Two ml aliquot of the extract was treated with following reagents to test the presence or absence of alkaloid.

Reagent	Positive result
Dragederoff's reagent	Orange or orange or red precipitate.
Mayers reagent	White precipitate or Turbidity

Test for Steroids and Sterols

a) Salkowski's Test

The extracts were dissolved in 1 or 2 ml of chloroform and 1 equal volume of concentrated sulphuric acid were added by the sides of the test tube. The upper layer turns red. Reveals presence of steroid and compounds in the extract.

Test for Flavonoids

a) One ml of the extract was treated with magnesium turnings and 1-2 drops of concentrated HCL. Formation of pick or red colour shows the presence of flavonoids.

b) One ml of the extract was treated with 1 ml of ferric chloride. The formation of brown color confirms the presence of flavonoids.

Test for Tannins and Phenolic compounds

a) One ml of the extract was treated with few ml of 5% neutral ferric chloride. A dark blue or bluish black colour product shows the presence of tannins.

b) One ml of the extract was treated with few ml of gelatin solution; a white precipitate reveals the presence of tannins and phenolic compounds.

c) One ml of the extract was treated with few ml of lead tetra acetate solution. A precipitate production shows the presence of tannins and phenolic compounds.

Test for Amino acids proteins

a) To one ml extract, 2 drops of freshly prepared 0.2% Ninhydrin reagent was added and heated. A blue colour develops indicating the presence of proteins.

b) The extracts were treated with 1.0 ml of 40% sodium hydroxide solution and 2 drops of 1% copper sulphate reagent. Appearance of violet colour indicates the presence of proteins.

Test for Carbohydrates

a) Fehling's test

The extract was treated with 5.0 ml of Fehling's solution and kept at boiling water bath. Formation of yellow or red colour precipitate indicates the presence of reducing sugars.

b) Benedicts test

To 1 ml the extract, added 5 ml of Benedict's solution and kept at boiling water bath. Red, yellow or green precipitate indicates the presence of reducing sugars.

Test for Cardio glycosides

Salkowski test

0.5 ml of the extract was dissolved in 2 ml of chloroform and sulphuric acid was carefully added to form a lower layer. A reddish brown colour at the interface indicates the presence of a steroidal ring.

Test for saponins

a) About 1 ml of alcoholic extract was diluted separately with 20ml of distilled water and shaken in a graduated cylinder for 15 minutes. A one cm layer of foam indicates the presence of saponins.

b) To 1 ml of the extract alcoholic vanillin solution and a few drops of concentrated sulphuric acid was added. A deep violet colour confirms the presence of saponins.

Test for fixed oils and fats

Spot Test

Press a small quantity of extract between two-filter papers. Oil stains on the filter paper indicates the presence of fixed oil.

Test for Terpenoids

a. Harizon test

To 1 ml of extract 2 ml of Tricholoroacetic acid (TCA) was added the formation of yellow to red precipitate shows the presence of terpenoids.

b. Lieberman test

To 1 ml of extract 3 ml of acetic acid and few drops of concentrated sulphuric acid was added. Colour change from red to blue indicates the presence of terpenoids.

EX. NO. 2

ESTIMATION OF TANNINS

AIM

To estimate the amount of tannins present in a given amount of plant extract.

PRINCIPLE

Tannins are wide spread in nature and probably in all plant materials. The polyphenolic compounds are divided into 2 main groups, hydrolysable and condensed. The estimation of tannin is based on the stoichiometric oxidation of molecules containing a phenolic hydroxyl group. Tannin reduces phosphomolybdic acid in alkaline condition to lower oxides of molybdenum producing a colored complex, the absorbance of which is measured at 700 nm.

REAGENTS

- Folin-denis reagent: 100 g sodium tungstate and 20 g phosphomolybdic acid was dissolved in 750 ml distilled water in a suitable flask and 50 ml phosphoric acid was added. The mixture was refluxed and was made for 2 hrs and make up to 1L with water and stored in brown bottle.
- 35% Na_2CO_3: The solution was allowed to stand for overnight, filtered through glass wool and used.
- Standard tannic acid solution (0.1 g/dl).

PROCEDURE

0.2 - 1.0 ml of standard tannic acid solution was pipetted out into a series of test tubes. To another test tube 0.5 ml of extract solution was taken. The volumes of all the tubes were made up to 3 ml with distilled water. 3 ml of distilled water was taken as blank. To all the tubes added 5 ml of 35% $Na_2 CO_3$ followed by the addition of 2.5 ml of Folin-denis reagent and incubated at room temperature for 30 minutes. The absorbance was read against reagent blank at 700 nm. From the standard graph the amount of tannin present in the sample was calculated.

TABULATION

S.No.	Solution		Volume of distilled water (ml)	Volume of 20% sodium carbonate (ml)	Volume of Folin's Dennis reagent (ml)	Incubate at room temperature for 30 minutes	Optical Density at 700nm
	Volume (ml)	Concentration (µg)					
Blank	-	-	3.0	5.0	2.5		
Standard S1	0.2	20	2.8				
S2	0.4	40	2.6				
S3	0.6	60	2.4				
S4	0.8	80	2.2				
S5	1.0	100	2.0				
Sample U1	0.5	-	2.5				
U2	1.0	-	2.0				

CALCULATION

The O.D reading ____ corresponds to ___µg of tannin

0.5 ml of sample contains = ___µg of tannin

1.0 ml of sample contains = ___ x1/0.5 µg of tannin

75ml of sample contains = ___x75/0.5x1000 mg of tannin

RESULT

1. The given plant extract contains of _____mg of tannin.
2. The given 0.5g of Unknown sample contains ____mg of tannin.

EX. NO. 3

ESTIMATION OF FLAVONOID

AIM

To estimate the amount of Flavonoids present in a given plant sample.

PRINCIPLE

Flavonoids reacts with aluminium chloride in ethanolic solution forms a yellow color which was read colorimetrically at 420 nm.

REAGENTS

- 2% aluminium chloride
- Ethanol
- Stock standard: 10 mg of quercetin/10 ml distilled water

PROCEDURE

A volume of 0.5 ml of 2% of $AlCl_3$ in ethanol solution was added to 0.5 ml of sample solution. After one hour incubation at room temperature, yellow color was developed. This was measured at 420 nm with UV-Visible spectrophotometer. A standard graph was prepared using the quercetin and the total flavonoid content was expressed as quercetin equivalent (mg/g).

TABULATION

S.No.	Solution		Volume of distilled water (ml)	Volume of aluminium chloride (ml)	Incubate at room temperature for 1 hour	Optical Density at 420nm
	Volume (ml)	Concentration (µg)				
Blank	-	-	1.0	5.0		
Standard S1	0.2	20	0.8			
S2	0.4	40	0.6			
S3	0.6	60	0.4			
S4	0.8	80	0.2			
S5	1.0	100	0			
Sample U1	0.5	-	0.5			
U2	1.0	-	0			

CALCULATION

The OD reading ____ corresponds to ____ µg of flavonoid.

Therefore, 0.5ml of sample contains = ____µg of flavonoid.

1 ml of sample contains = ___ x 1/0.5 µg of flavonoid

50 ml of sample contains = ___ x 50/1x1000 mg of flavonoid

RESULT

The given 50 ml of unknown sample contains ____mg of flavonoid.

EX. NO. 4

ESTIMATION OF CHLOROPHYLL

AIM

To estimate the amount of chlorophyll present in a given green leaf sample.

INTRODUCTION

The chlorophyll is the essential components for photosynthesis and occurs in chloroplast as green pigments in all photosynthetic plant tissue. They are bound loosely to proteins but are readily extracted in organic solvents such as acetone or ether. Chemically each chlorophyll molecule contains a porphyrin nucleus with a chelated magnesium atom at the center and the long chain hydrocarbon side chain attached to a carboxy acid group. There are atleast five types of chlorophyll plants chlorophyll 'a' and chlorophyll 'b' occurs in higher plants, ferns, mosses, chlorophyll c, d and e are only found in algae & in certain bacteria.

PRINCIPLE

Chlorophyll is extracted in 80% acetone & absorption exist in 630nm & 645nm are read in spectrophotometer, using the absorption coefficient the amount of chlorophyll is calculated.

MATERIALS REQUIRED

Dilute analytical grade acetone to 80% acetone (pre-chilled).

PROCEDURE

1. Weigh 1g of finely cut & mixed representation sample of leaf or fruit tissue into a clean mortar.
2. Grind the tissue to find pulp with the addition of 20ml of 80% acetone.
3. Centrifuge 5000rpm for 5 minutes & transfer the supernatant to 100ml volumetric flask.
4. Repeat the procedure until the residue is colourless. Wash the mortar & pestle thoroughly with 80% acetone & collect the clear washing in the volumetric flask.
5. Make up the volume to 100ml with 80% acetone.
6. Read the absorbance of solution against the solvent blank (80% acetone) at 645, 663nm.

CALCULATION

The amount of chlorophyll present in the extract (mg) chlorophyll per gram tissue is calculated as follow

mg chlorophyll 'a'/ gram tissue = 12.7(absorbance at 663nm)-2.69(absorbance at 645nm)

× V/1000 × W

mg chlorophyll 'b'/ gram tissue = 22.9 (absorbance at 645nm)-4.68(absorbance at 663nm)

× V/1000 × W

mg total chlorophyll/ gram tissue = 22.9 (absorbance A at 645nm)-4.68(absorbance B at 663nm)

× V/1000 × W

Where,

A- Absorbance at specific wavelength

V- Final volume of chlorophyll extract in 80% acetone

W- Fresh weight of tissue extract

RESULT

1. The amount of chlorophyll 'a' present is ---------- mg/cumm/g tissue
2. The amount of chlorophyll 'b' present is ---------- mg/cumm/g tissue
3. The amount of chlorophyll total present is ---------- mg/cumm/g tissue

EX. NO. 5

ESTIMATION OF TOTAL PHENOL

AIM

To estimate the **total phenol content** present in a given sample.

PRINCIPLE

Phenols react with phosphomolybdic acid of Folin-ciocalteau reagent in alkaline medium and produce a blue colored complex (molybdenum blue) that can be estimated colorimetrically at 650 nm.

REAGENTS

- 20% sodium carbonate
- Folin-ciocalteau reagent
- Standard gallic acid: 10 mg of gallic acid/10 ml of distilled water
- 80% ethanol

PROCEDURE

An aliquot of the sample extract (0.1 ml) was mixed with distilled water (3 ml) and 0.5 ml of Folin-ciocalteau reagent was added. After 3 min, 2 ml of 20% sodium carbonate was added and mixed thoroughly. The tubes were incubated in a boiling water bath exactly 1 min. It was then cooled and absorbance was measured at 650 nm using spectrophotometer against the reagent blank. A calibration curve was constructed with different concentration of gallic acid (0.01- 0.1 mM) as standard was prepared and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

TABULATION

S.No.	Solution		Volume of distilled water (ml)	Volume of Folin's Ciocalteau reagent (ml)	Incubate at room temperature for 3 minutes	Volume of Sodium carbonate (ml)	Incubate in boiling water bath for 1 minute and then cooled	Optical Density at 650nm
	Volume (ml)	Concentration (µg)						
Blank	-	-	3.0	0.5		2.0		
Standard S1	0.2	20	2.8					
S2	0.4	40	2.6					
S3	0.6	60	2.4					
S4	0.8	80	2.2					
S5	1.0	100	2.0					
Sample U1	0.1	-	2.9					
U2	0.1	-	2.9					

CALCULATION

The OD reading ____ corresponds to ____ µg of phenol content.

Therefore, 0.1 ml of sample contains = ___µg of phenol content.

100 ml of sample contains = ___ x100/0.1x1000 mg of phenol

50 ml of sample contains = ___x50/1000 mg of phenol

RESULT

The given 1 g of sample contains _____ mg of phenol.

EX. NO. 6

MICROBIOLOGY

ISOLATION OF BACTERIA FROM SOIL BY SERIAL DILUTION

AIM

To determine the number of bacteria, actinomycetes and molds present in the soil.

PRINCIPLE

Different kinds of microorganisms in large number are present in soil. The type of microorganism that predominantly present in soil depends on some type, moisture, pH and other environmental factors. By single method it is impossible to count all microorganism since such a 6reat variability and type of organism present in soil. Therefore it is necessary to use different type of media for growth of microorganisms {~erial dilution technique and plating on nutrient media can be used to determine the number of viable microorganisms)

MATERIALS REQUIRED

Nutrient agar, Glycerol yeast extract agar; Glucose peptone acid agar, Petri plates, Pipettes, Test tubes, Garden soil samples.

PROCEDURE

1. Prepare nutrient agar, glycerol yeast extract agar and glucose peptone acla-aqar and pour them in sterile petriplates. Allow to solidify and label them.
2. Take 1 gm of soil and suspend it in 10 ml of water. Allow to stand on work table for 5 minutes.
3. Make dilution blanks with water in sterile test tubes (4,5 ml tube) and label them as 10^{-1} 10^{-2} ... 10^{-7}
4. Serially dilute the soil suspension in water using the dilution tubes.
5. Take 0.1 ml sample each from 10-4, 10-5, 10-6, 10-7 dilutions and plate them on nutrient agar plates. (for counting bacterial colonies)
6. Take 0.1 ml from 10-3, 10-4, 10-5, 10-6 dilution and plate them on glycerol yeast extract agar. (for counting actinomycetes colonies)
7. Take 0.1 ml each from 10-2, 10-3, 10-4 and 10-5 and plate them on glucose peptone acid agar. (For counting mold colonies)

8. Incubate the plates at room temperature for 3 to 7 days and observe the plates every day.

9. The colonies in the plates and calculate the number of bacteria (nutrient agar), Actinomycetes (Glycerol yeast extract agar), Molds (peptone glucose acid agar) present in a gram of soil taking into consideration of the dilution factors.

RESULT

Total number of bacteria present in the soil is determined as _____.

EX. NO. 7

ISOLATION OF PURE CULTURE – STREAK PLATE, POUR PLATE, SPREAD PLATE METHODS

OBJECTIVE

To learn the pure culture technique and isolate pure culture of bacteria.

BACKGROUND

The microbial population in our environment - air, soil, water - is large and include many" species of bacteria, fungi and algae. A study of the microorganisms in these habitats requires knowledge of the specific microbes present. This requires techniques for preparing species as pure cultures from the mixed population.

Pure culture represents a population of organisms of a single species in the absence of living cells of any other species. There are various techniques whereby the different species in a natural population can be isolated and grown as pure culture. They are a) streak plate technique, b) pour plate technique and c) spread plate technique.

Materials required

Nutrient agar, Nutrient broth, Petriplates, Pipettes Dilution blank, L-rod, tubes, Vortex mixer.

a) STREAK PLATE TECHNIQUE

- Use the nutrient agar plates prepared as earlier.
- Sterilize the inoculation needle by flaming it red hot and allow it to cool for 30 seconds.
- Holding the culture tube in your left hand, take the tube near flame and remove cotton with your right hand and flame the mouth of the tube for a few seconds.
- Touch the culture with the needle loop and take the culture.
- Place it on the agar plate and streak it on the plate once.
- Resterilize the loop and cool it as above and take the culture from one end of the plate to other end and complete the streak as indicated.

b) POUR PLATE TECHNIQUE

- Liquefy the nutrient agar in the tubes by heating them in water bath.
- Cool the tubes to 45°C and hold at this temperature until ready to pour into the plate.
- Label the tubes and corresponding Petridishes.
- Serially dilute the given mixed culture and from each dilution take 1 ml of the sample.
- Mix the sample with agar medium by gently rotating the tube between your palms.
- Pour the contents of the tubes into the corresponding labelled petri plates and allow to solidify.
- Incubate the plates in inverted position at room temperature.

c) SPREAD PLATE TECHNIQUE

- Use the nutrient agar plates as prepared in your earlier experiment.
- Serially dilute the given culture as given in section b (pour plate technique).
- Label the nutrient agar plates corresponding to the dilutions.
- Place 0.1 ml from the each dilution in seperate nutrient agar plates.
- Sterilize the 'L' rod with alcohol and then by flaming it.
- Cool the rod and place the rod gently on the surface of the agar. Spread the sample over the surface of the agar medium by rotating the petriplate manually or by placing it on the rotating plate disc.
- Incubate the plates in inverted position at the room temperature.

Observe the well seperated colonies from these different techniques. Pick up isolated colonies with the sterile inoculation needle and inoculate into the liquid medium. Incubate them as before. After sufficient growth make wet mount of the cultures and observe under microscope. If the cultures are pure (axenic) they may be individually transferred to agar slants and labelled.

EX. NO. 8

COLONY MORPHOLOGY – COLONY COUNTING

AIM

To observe the features of colonies and to enumerate the number of colonies on agar plates.

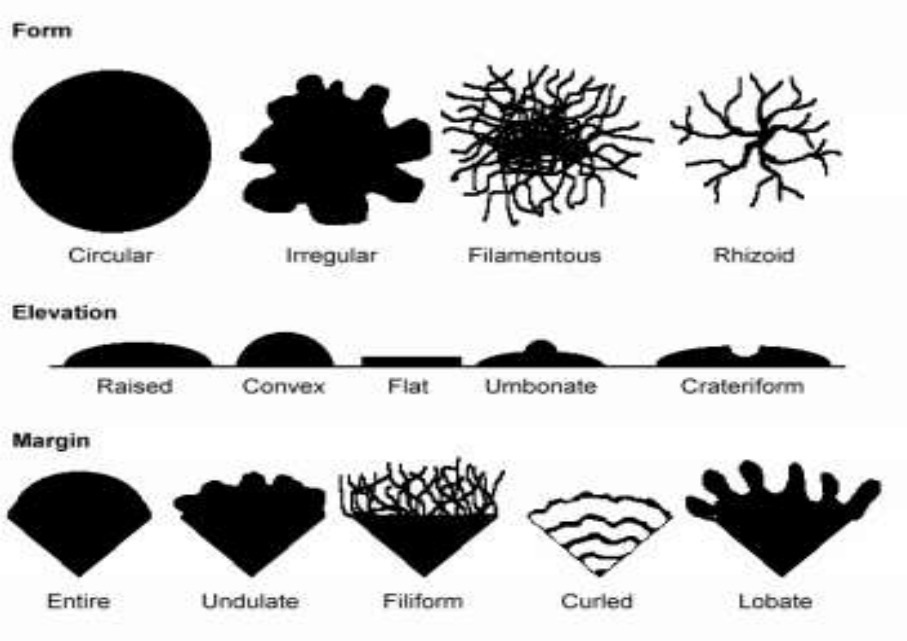
PRINCIPLE

The major feature of bacterial strain is their appearance following growth on various media. The abundance of growth, the size and colour of the colonies provide useful clues for identification. To determine the growth characteristics of a bacterial stain, it is customary to observe the features of colonies on plates and in broth cultures. Generally, a loop is used when the inoculums is a liquid culture. After inoculation of the medium and subsequent inhibition, the cultural characteristics can be determined. The main features of growth on media are summarized.

OBSERVATION

On a given medium, a colony's shape, color, consistency, surface appearance and size - for a given incubation time - are often characteristic, and these are often of use in the identification of particular bacterial strains. The full description of a colony can be very detailed. Thus e.g. the elevation of a colony may be flat, low convex, domed unbonate etc., its edge maybe entire [circular or unbroken], crenate [scalloped], lobed or fimbriate; its texture may be butyrous friable or mucoid; its surface may be matt or glossy; it may be whitish or pigmented or it may contain a dye taken up from the medium, or it may release water soluble pigment into the medium. The colonies of certain bacteria e.g. Bacillus can migrate across the surface of a culture plate, the tract of such movement is often marked by lines of bacterial growth which arise from the cells left behind by the migration colony. An interesting feature of certain bacterial colonies is the so-called smooth-rough variation. In many types of bacteria, some type of S-R variation is responsible for a change in the cell-surface composition, which occurs spontaneously during in vitro or in vivo growth. S-R variation was first recorded in enterobacteria, in which smooth

[glossy] colony may be formed on primary isolation, and rough [dull] colonies may develop on subcultures.



Basic Colony Morphology Types

EX. No. 9

STAINING TECHNIQUES- SIMPLE, DIFFERENTIAL, SPORE, AND FUNGAL STAINING

AIM

To learn the various staining techniques to study morphology of microorganisms.

BACKGROUND

Staining is the method of artificially producing colour in micorogranisms to allow for the visualization under the microscope; Stains are employed not only to make the organisms visible but also understand their structure and chemical nature.

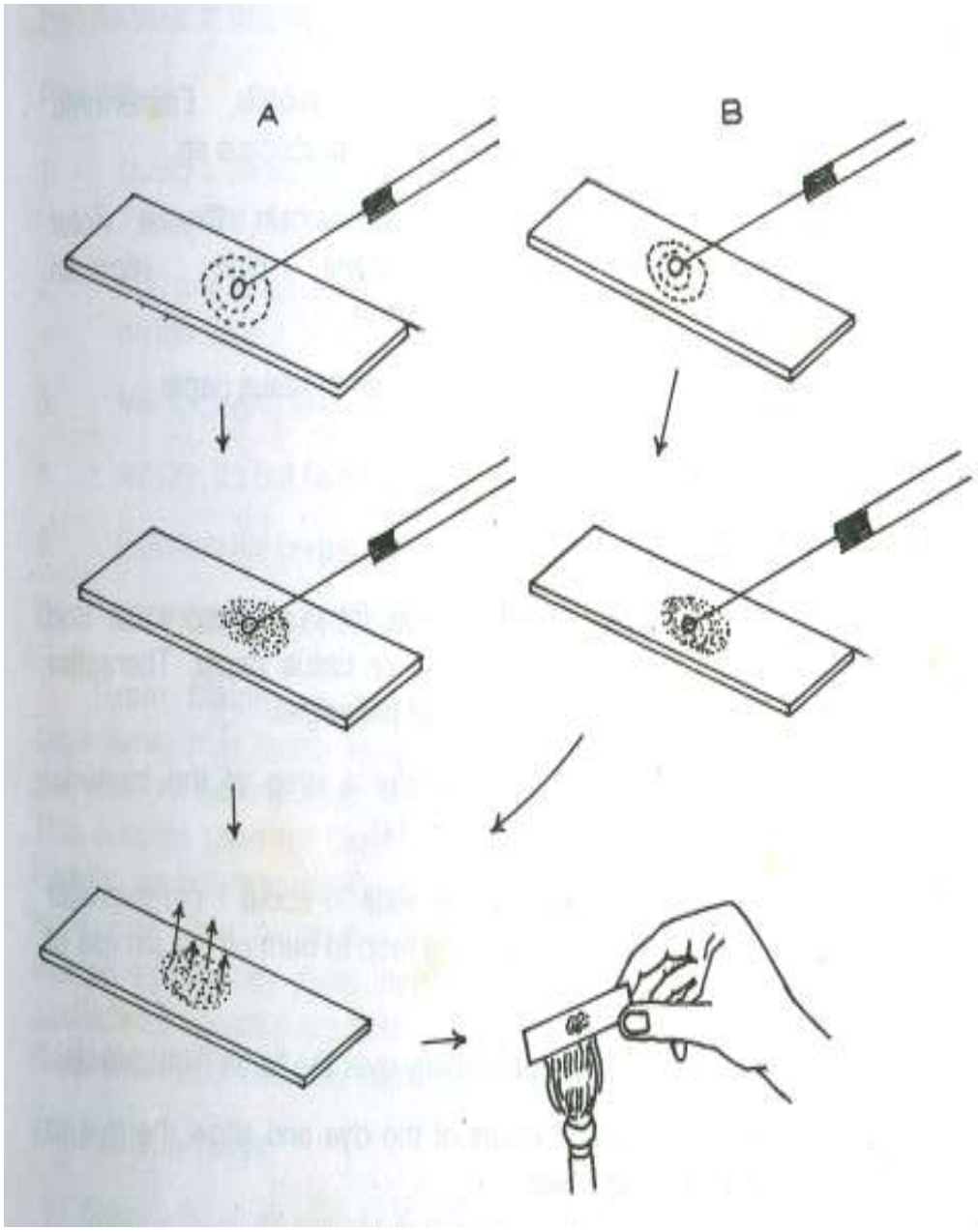
Dyes or stains are synthetic chemical products of the aniline type. They can be divided into acid dyes and basic dyes with respect to their colour property in the anionic and cationic form. An acid dye is the salt of a coloured acid while a basic dye is the salt of a coloured base. For example, Crystal violet, Methylene blue are basic dyes and Acid fuchsin and Erythrosin are acid dyes. Acid dyes have stronger tendency to combine with the cytoplasm whereas basic dyes have greater affinity for the nuclear region of the cells. Basic dyes are generally used in bacteriological studies.

Knowledge of the basis for the specificity of dyes is important to understand differential staining reactions. For example in Gram's staining, the primary dyes are of the basic triphenyl methane type (crystal violet or methyl violet). Alkaline aqueous solution of these dyes readily penetrate the bacterial cells or yeast and stain them dark blue. If the stained cells are washed extensively with water or dilute acid much of the primary dye is removed. Instead, if the cells are next treated with iodine solution which acts as a mordant a water insoluble dye complex is produced. Then the cells are then treated with an organic solvent (alcohol or acetone) the dye iodine complex is quickly dissolved and tends to be removed from the cell' contents whereas blue colour persists longer in the cell walls of Gram positive bacteria.

Application of an aqueous counter stain results in staining the decolourlzed Gram negative cells whereas Gram positive cell wall do not react with the counter stain. Evidently the essential difference between Gram positive and Gram negative microorganisms is in the chemistry of the cell wall. However, some of the environmental factors can also influence the

staining property of the cells. They are a) Age of the culture; b) pH of the environment (medium). The response to Gram Stain is of value in classifying bacteria.

Fig: Preparation of culture slide



- From the liquid culture B. From the solid medium

EX. NO. 9A

SIMPLE STAINING

AIM

To identify the morphology and arrangement of bacterial cell by simple staining.

PRINCIPLE

When bacteria smear are stained with a reagent then it is called a simple staining. The basic dye such as crystal violet on methyl blue is used. The smear was flooded with crystal violet for 20-60 seconds. On methyl blue for 1-2 minutes. The positive charged dye (basic dye) was attracted to the bacterial cell wall, nucleic acid, cytoplasm which has a negative charged and staining takes place. This is effective for vegetative cells and the stains do not penetrate spore.

MATERIALS REQUIRED

- Cultures (24 hrs. old): *Bacillus subtilis*, *Escherichia coli*, *Streptococcus sp*.
- Stains : Carbol fuchsin, Crystal violet, Methylene blue, Nigrosin solution
- Clean glass slides, Microscope and clean cloth or tissue paper.

PROCEDURE

I. Positive Staining

1. Clean the slides in any detergent solution. Rinse with tap water and wipe the slides. Dry with a clean cloth or tissue paper. Thereafter handle the slides only by grasping them at the edges.
2. By means of a sterile culture loop, transfer a drop of the bacterial suspension to the centre of the slide.
3. Spread the drop over the centre of the slide to about 1 crn diameter and allow the film to air dry. Reflame the loop to burn off the excess of inoculam in it. Cool.
4. Fix the smear by passing the slides rapidly over the flame (film side up).
5. Flood the smear with several drops of the dye and allow the dye to remain for the following intervals.

Carbolfuchsin - 15-30 sec.
or
Crystal violet - 30-45 sec.
or

Methylene Blue - about 3 mins.

6. Wash the slide in a gentle stream of tap water to remove excess of . stain. Air dry.
7. Examine the preparation with the oil immersion objective.

RESULT

The morphology of organism was found to be _____

EX. No. 9B

GRAM (DIFFERENTIAL) STAINING

AIM

To identify the morphology og bacterial all and to differential two principle groups of bacteri is gram positive and gram negative.

PRINCIPLE

Gram Staining is employed to visualize and differentiate between organisms. It is useful in presumptive identification of organisms before carrying out several other tests such as seroloqlcal, and biochemical tests. The cultures used for Gram stain reaction should be less than 24 hr. old. Reason for failure to stain positively are age of culture, low pH of medium or both. True Gram negative organism will not become gram positive although increasing alkalinity make them appear as positive. Increase in acidity may cause Gram positive organism to appear Gram negative. Cocci are generally Gram positive (except Neisseria).

MATERIALS REQUIRED

Glass slides, 24 hrs old culture of *Escherichia coli* and *Bacillus subtilis*, Crystal violet, Potassium Iodide/Iodine Solution, Acetone-Alcohol, Safranin.

PROCEDURE

1. Prepare the smear on the slides with the bacterial cultures as done for the simple positive staining method.
2. Stain it for one minute with crystal violet solution (Gram's stain)wash it in tap water.
3. Apply the Iodine solution (Mordant) for 1 min. Wash in tap water.
4. Decolourize with alcohol by adding dropwise on the tilted slide until all free blue colour has been removed (20-30 sec). Wash it in tap water.
5. Flood the siide with safranin (counter stain) for one minute. Wash it in tap water and air dry.
6. Examine the stained smear under the oil immersion objective to determine which organism is Gram positive (Violet colour) and which is Gram negative (Pink colour).

RESULT

The morphology of organism was found to be _____.

EX. No. 9C

ENDOSPORE STAINING

AIM

To perform spore staining and to differential endospore from vegetative cell.

PRINCIPLE

Spore are metabolically inactive spores may be exospores and endospores. Enospores are produced within a cell the spores in case of bacteria is meant to live at unfavorable condition (ie) it leads to a dormant state the spores are highly resistant to disfecting agents and UV rays to due to the presence of thick layers called spore coats. The calcium dipicoline acid present in the spore wall is responsible for high resistance. Two different strains are used.

MATERIALS REQUIRED

Bacillus subtilis slants (24 hr. culture), Stain. a) Malachite green (5% w/v aqueous solution) and b) Mercurochrome (5% w/v aqueous solution).

PROCEDURE

- Prepare a smear of *Bacillus subtilis* on a clean glass slide. Air dry and fix with heat.
- Flood the slide with Malachite green and place a cut piece of paper to wetting over the smear, so that it completely covers the smear and soaks up most of the stain. The toweling should be saturated with the stain throughout the staining process.
- Gently heat the slide until the stain begins to steam and keep it for 5 mins.
- Remove the paper towel and wash the slide gently with water until all excess stain is removed.
- Counter stain with basic fuchsin for 30 sec.
- Wash with water. Blot it and air dry.
- Examine the slide using oil immersion objective. Red coleured cells containing green spores can be seen.

RESULT

The morphology of organism was found to be _____.

EX. No. 9D

FUNGAL STAINING

AIM

To identify the fungi by lacto phenol cotton blue staining

PRINCIPLE

The lactophenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi and is simple to prepare. The preparation has three components: phenol, which will kill any live organisms; lactic acid which preserves fungal structures, and cotton blue which stains the chitin in the fungal cell walls.

MATERIALS REQUIRED

- Slide
- Inoculation loop
- Bunsen burner
- Fungal Culture
- Cover slip
- LPCB stain

PROCEDURE

- Take a clean dry glass slide
- A drop of LBCB stain was placed in the centre of the slide
- With the help of inoculation loop a small portion of colony was scratched and placed in the drop of stain
- Then it was teased into small bits with the help of teasing needle
- A cover slip was placed on top and gentle pressure was applied for even spreading
- It was then observed under the microscope.

RESULT

The morphology of organism was found to be _____.

EX. No. 10

ANTIBIOTIC SENSITIVITY TEST (DISC METHOD)

AIM

To determine the antibiotic sensitivity of bacterial strain and to show the minimal inhibitory concentration of antibiotic to bacterial strain.

MATERIALS REQUIRED

Antibiotic disc, nutrient agar, bacterial culture, petriplates.

PROCEDURE

1. Select and label the culture that are to be used for antibiotic sensitivity assay (*E.coli, Aspergillus, Pseudomonas*)
2. Prepare nutrient agar plates.
3. Aseptically taken a swab of test culture and inoculate on the surface of nutrient agar plate completely. Allow at least 40 minutes to dry.
4. Take a forceps and sterilize the tip by dipping in alcohol and then flame it and allow it to cool.
5. Carefully take the antibiotic disc to the agar plate. Gently press the disc to give a better contact with the agar.
6. Place at least four different antibiotic disc at the same distance apart from each other in the agar plate.
7. Incubate the plate in their inverted position for 16-18 hours at room temperature.
8. Observe the zone of inhibition around the antibiotic disc.
9. Indicate whether the test organisms is resistant.

RESULT

All the test organisms were sensitive towards the different antibiotics used.

EX. No. 11

ESTIMATION OF BACTERIA- GROWTH CURVE OF BACTERIA AND GENERATION TIME

AIM

To perform growth curve and find the generation time of bacteria

PRINCIPLE

Microbial population growth studies require inoculation of viable cells into a sterile broth medium of the culture under optimum temperature, pH and gaseous condition. Under these conditions the cell will reproduce rapidly and the dynamics of the microbial growth curve within is constructed by plotting the increase in the cell member versus time of incubation. The curve can be used to determine the stage of growth cycle. It also facilitates measurements of cell member and rate of growth of a particular organism under standardised conditions expressed by its generation time, the time required for a particular population to double in number.

STAGES OF TYPICAL GROWTH CURVE

(a)Lag Phase:

During this stage the cells are adjusting to their new environment the cellular metabolism was accelerated, resulting in rapid biosynthesis of cellular macromolecules primarily enzyme in the preparation for the next phase of cycle. Although the cells are increasing in size there's no increase in number.

(b)Logarithmic or Log Phase

Under optimum nutritional and physical condition, the physiological robust cells reproduce at a uniform rate of budding. Thus there is a rapid exponential increase in population while doubles regularly until a maximum of cells are reached.

(c)Stationary Phase

During this stage, the number of cells undergoing division is equal to the number of cells that are dying. Therefore there's no further increase in cell member and population is maintained at its maximum level for a period of time. The primary factor responsible for this phase are the depletion of some essential metabolites and accumulation of toxic acid or alkaline product in the medium.

(d) Decline or Death Phase

Because of counting depletion of nutrients and build up of metabolic waste, the microorganisms dying at a rapid and uniform rate. The decrease in population is closely parallel to its increasing log phase.

GROWTH CURVE CAN BE DETERMINED BY FOLLOWING METHODS

DIRECT MICROSCOPY

The uses hemocytometes to count the member of cells developed at 60 minutes time intervals as an index of increasing cellular mass.

a) **Turbidity method:** This uses spectrophotometer measurements of developing turbidity at 60 minutes interval as an index of increasing cellular mass.
b) **Viable count:** This method involves serial dilution of bacterial suspension in sterile water blanks which serves as diluents of known volume. Once diluted, the suspensions are placed on nutrient medium.

PROCEDURE

1. Nutrient broth was prepared and 500 ml of medium was dispersed into series of 100 ml conical flask and sterilized at 121˚C, 15 it's for 15 minutes.
2. Then the flask were aseptically inoculated with 1 ml of overnight *E.coli* culture and placed in a shaker for agitation and aeration.
3. The flasks were removed at an interval of 30 minutes and the samples were transferred asceptically to a clean cuvette using a sterile.
4. Optical density was read using spectrophotometer at 620 nm and the readings were tabulated.
5. Then the readings were plotted on a graph by taking OD value on y-axis and incubation on x- axis and *E.coli* growth curve was drawn.

DETERMINATION OF GENERATION TIME

1. Indirect determination is made by simplest extrapolation from the log phase.
2. Two points were selected on the OD scale that represents a doubling of turbidity.
3. Using a rules the OD points were extrapolated by drawing a line between each of the points on the coordinate and plotted the growth curve.

4. The perpendicular lines were drawn from end points on growth curve to their respective time intervals on the abscissa, generation time was determination using the formula:

Generation time = T_0D_2 = T_0D_1

GROWTH CURVE OF BACTERIA

S. No	Incubation Time (minutes)	Optical Density (620 nm)
1.		
2.		
3.		
4.		
5.		
6.		
7.		

CALCULATION

Generation time = T_0D_2 = T_0D_1

RESULTS

The generation time of the *E.coli* culture is ---------- minutes.

EX. No. 12

IDENTIFICATION OF MICROORGANISMS – BIOCHEMICAL TESTS (IMVIC TEST)(GROUP EXPERIMENT)

IMVIC reactions are a set of four useful reactions that are commonly employed in the identification of members of family enterobacteriaceae. The four reactions are: Indole test, Methyl Red test, Voges Proskauer test and Citrate utilization test. The letter "i" is only for rhyming purpose.

(a) INDOLE TEST

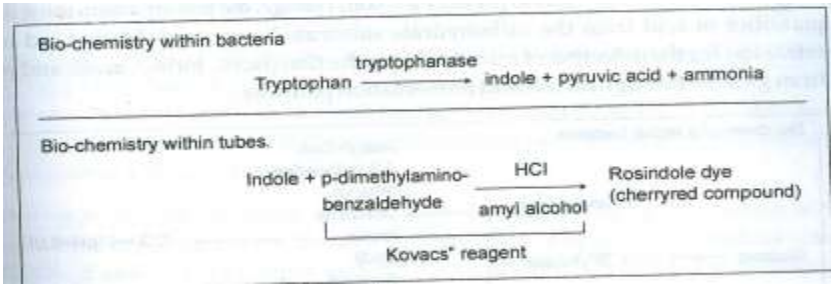
AIM

- To determine the ability of microorganisms to degrade the aminoacid tryptophan
- To determine the ability of microorganisms to produce indole.

PRINCIPLE

Tryptophan is an essential aminoacid that can undergo oxidation by the enzymatic activities of some bacteria. Conversion of tryptophan into metabolic end product is mediated by the enzyme tryptophanase.

The presence of indole is detectable by adding Kovac's reagent, which produces a cherry red reagent layer. The colour is produced by the reagent, which is composed of p, dimethylaminobenzaldehyde, butanol and hydrochloric acid. Indole is extracted from a medium into the reagent layer by the acidified butanal component and forms a complex with the P-dimethyl amino benzoldehyde, yielding a cherry red colour (Rosindole dye).



Cultures producing a red reagent layer following the addition of kovacs reagent are indole positive. The absence of red colouration demonstrates that the substrate tryptophan is not

hydrolysed and indicates an indole negative reaction.

MATERIALS REQUIRED

SIM agar or Peptone water, Kovacs indole reagent, Test tubes, cotton, conical flask, pH paper/ pH meter etc.,

Quality Control

Positive control: *Escherichia coli*

Negative control: *Klebsiella pneumoniae*

PEPTONE BROTH:

- Peptone - 20g
- NaCl - 5g
- Distilled water - 1000ml
- pH - 7.4

KOVACS INDOLE REAGET:

- Amyl 1Iso amylalcohol - 150ml
- P dimethyl aminobenzaldehyde - 10g
- Concentration HCl - 50 ml

PROCEDURE

- Prepare SIM agar or peptone water medium and sterilize at 121°C for 15 minutes . Inoculate the medium with test organism by using suitable technique.
- Incubate the medium at 370C for 24 hours .
- Look for growth after 24 hours and add one mL of kovacs indole reagent. Observe colour change and record the results.

OBSERVATION

Cherry red colour formation indicates positive result. Yellow or other colour change indicates negative results.

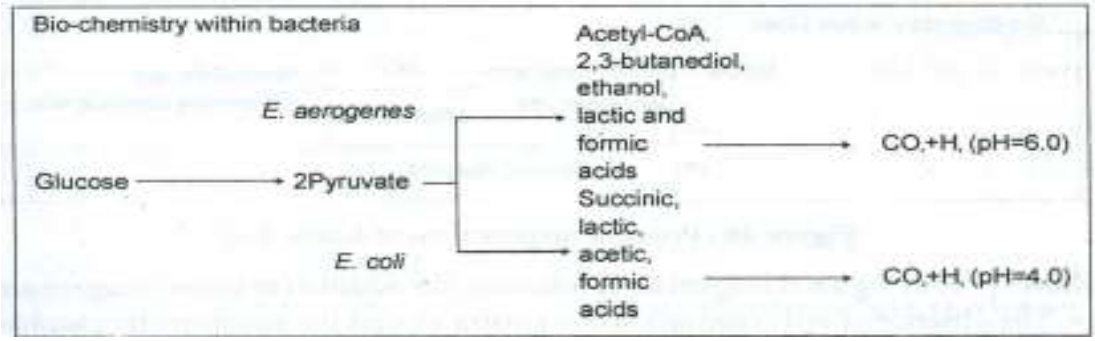
(b) METHYL RED TEST

AIM

To determine the ability of micro organisms to oxidize glucose with the production of high concentration of acid end products.

PRINCIPLE

Methyl red is a pH indicator with a range between 6 (yellow) and 4.4 (red). The pH at which methyl red detects acid is considerably lower than the pH of other indicators used in bacteriological culture media. Thus to produce a colour change, the test organism must produce large quantities of acid from the carbohydrate substrate being used. Methyl red test is a quantitative test for the detection of mixed acid production (lactic, formic, acetic and pyruvic acids) from glucose through mixed acid fermentation pathway.



MATERIALS REQUIRED

MR- VP broth, Methyl red pH indicator, Test tubes, Inoculation loop, Cotton etc.

QUALITY CONTROL

Positive control: *Escherichia coli*

Negative control: *Klebsiella pnemoniae*

PROCEDURE

- Prepare MR- VP broth in test tubes and sterilize at appropriate temperature
- Inoculate the test organisms using sterile technique
- Incubate tubes at 3J70C for 24- 48 hours.

- At the end of incubation, add 5 drops of methyl red indicator directly to the medium .
- Gently shake the medium.
- Observe for colour change.

Observation

Colour change is observed

RESULT

Positive -red or pink colour

Negative -yellow colour

(c)VOGES PROSKAUER TEST

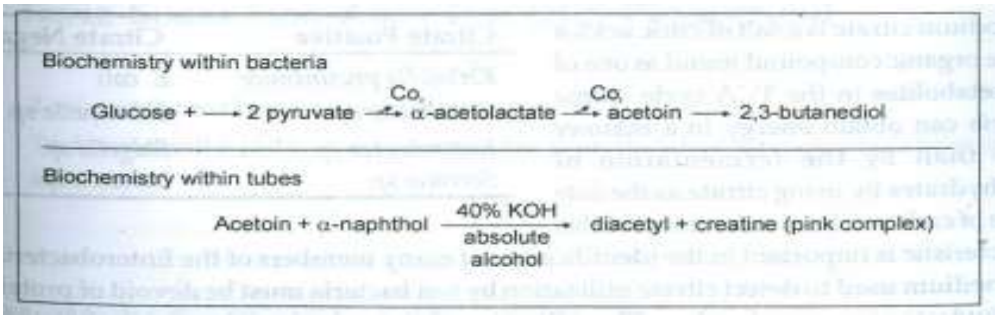
AIM

To differentiate enteric organisms.

PRINCIPLE

Voges and Proskauer are the two microbiologists working at the beginning of the 20th century. They first observed red colour reaction produced by appropriate culture media after treatment of potassium hydroxide. It was later discovered that the active product formed after bacterial metabolism is acetyl methyl carbinol (acetoin) a neutral reacting end product. It is a product of butylene glycol pathway.

Pyruvate is formed during the fermentative degradation of glucose, which is further metabolized by bacterial enzymes through butylene glycol path way and produce acetoin. In the presence of atmospheric oxygen and 40% KOH, acetoin is converted in to diacetyl and creatine. Alpha naphtol serve as a catalyst to bring out a red complex.



Material Methods

MR-VP broth, a napthol, Potassium hydroxide, Test tubes, Inoculation loop, Cotton etc.

Quality Control

Negative control: *Escherichia coli*

Positive control: *Klebsiella pnemoniae*

PROCEDURE

- Prepare MR-VP broth in test tubes and sterilize at 121°C for 10 minutes.
- Inoculate the test organisms using sterile technique.
- Incubate tubes at 3?'C for 24- 48 hours
- At the end of incubation, add 0.5 mL of a napthol, followed by 0.2mL of KOH.
- Shake the tube gently to expose the medium to atmospheric oxygen and allow the medium to stand for 10-15 minutes.
- Observe colour change and record the results.

Observation

Colour change was observed

RESULT

Positive -red or brown colour

Negative -black or pale yellow colour

(d) CITRATE UTILIZATION TEST

AIM

- To differentiate bacteria
- To detect the ablility of bacteria to utilize citrate as a carbon source

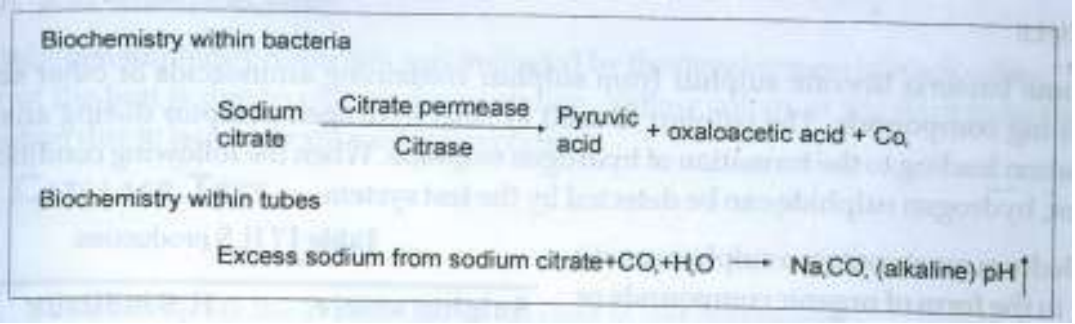
PRINCIPLE

Sodium citrate is a salt of citric acid, a simple organic compound round as one of the metabolites in the T'~A cycle. Some bacteria can obtain energy in a manner other than by the fermentation of carbohydrates by using citrate as the sole source of carbon. The measurement of this characteristic is important in the identification of many members of the Enterobacteriace Any medium used to detect citrate utilization by test bacteria must be devoid of protein carbohydrate as source of carbon. The utilization of citrate by a test bacterium is detected the

production of alkaline by products. The medium includes sodium citrate, an anion, as sole source of carbon and ammonium phosphate as a sole source of nitrogen. Bacteria that citrate also extract nitrogen from the ammonium salt, with the production of ammonia, lea . to alkalization of the medium from conversion of the NHt to NH_40H

Table 16 Quality control

Citrate Positive	Citrate Negative
Klebsiella pneumoniae	*E. coli*
Citrobacter sp.	*Salmonella sp.*
Enterobacter sp.	*Shigella sp.*
Serratia sp.	*Yersinia sp.*



MATERIALS REQUIRED

Test tubes, Conical flask, inoculation loop, cotton plug, Simmons citrate agar, test organisms etc.,

SIMMON CITRATE AGAR MEDIUM

- Nacl -5g
- $MgSo_4$ - 0.2g
- $NH_3H_2PO_4$ -1g
- K H_2PO_4 -1g
- Sodium citrate - 5g
- Agar - 20g

- 0.2% Bromothiynol Blue - 40ml
- Distilled water -1000ml
- pH - 6.8

PROCEDURE

- Prepare simmons citrate medium and poured in test tubes.
- Sterilize the medium at 121°C for 15 minutes.
- Pick a well-isolated colony from the surface of a primary isolation medium and inoculated as a single streak on the slant surface of the citrate agar tube.
- Incubate the medium at *3J1OC* for 24 hours.
- Observe colour change after incubation period.

RESULT

A positive test is represented by the development of a deep blue colour within 24-48 hours, indicating that the test organism has been able to utilize the citrate contained in the medium, with the production of alkaline products. A positive test may also be read without a blue colour if there is visible colonial growth along the inoculation streak line.

EX. No. 13

MICROBIOLOGY OF POTABLE WATER

AIM

To examine the presence of microbes in water and relate it to the portability of water.

PRINCIPLE

Water which receive human and animal wastes which form a primary source of waterborne disease). Direct testing procedures capable of detecting and quantifying the full spectrum of pathogens and identifying their sources are time consuming process because of their variable occurrence, survival rate and origin. Hence an indicator system has been identified as the best method for evaluating the microbiological quality of water.

MATERIALS REQUIRED

Lactose broth (double strength and single strength), Test tubes, Durham tubes, Pipettes, EMB agar, Nutrient agar, Brilliant green, Phenol red.

PROCEDURE

(i) Viable cell count

- Choose appropriate dilutions to get 30-300 colonies in a plate.
- Mix 0.1 ml. of diluted sample in to 15-20 ml of sterilized nutrient agar medium kept at 45°C.
- Rotate carefully to mix the medium and inoculum and pour it in the petridish. After it solidifies invert the plates and incubate at 3t'C for 24 hrs.

The Plate with 30-300 colonies is counted and multiplied by the dilution factor used.

RESULT: The bacterial density is expressed in number per milliliter of the sample.

(ii) Membrane filter technique

- Set up a membrane filter apparatus with s sterile membrane filter (0.45,um).
- Filter 100 ml of water sample (Depending on the number of coliforms present in the sample, the volume of sample to be filtered can be altered).
- Remove the membrane from the apparatus with sterile forceps and carefully place on surface of EMB agar plate.

- lncubate at 3f>C for 24 hrs.
- Count the number of typical and atypical colonies formed.
- Compare the result with the earlier experiment.

RESULT

The Number of colonies in one mille liter of water: _____

Ex. No. 14

ISOLATION, CHARACTERIZATION AND PURIFICATION OF MICROBIAL ENZYMES AMYLASE

AIM

To isolate and estimate the extracellular enzyme amylase.

PRINCIPLE

The reducing sugars produced by the action of alpha and beta amylase react with dinitrosalicylic acid and reduce it to a brown colour product, nitroamino salicyclic acid

MATERIALS REQUIRED

1. Sodium acetate buffer - 0.1N, pH-4.7.
2. Starch (1% solution):

Prepare a fresh solution by dissolving 1gm starch in 100 ml acetate buffer. Slightly warmed if necessary

3. Dinitro salicyclic acid reagent:

Dissolve by stirring 1g of dinitrosalicylic acid, 200mg crystalline phenol and 50mg of sodium sulphite in 100ml 1%sodium hydroxide. Store at 4^0C. Since the reagent detroit due to sodium sulphite if long storage is required, sodium sulphite may be added at the time of use.

4. 40% Rochelle salt solution (Potassium sodium tartarate)
5. Maltose solution:

Dissolve 50 mg maltose in 50ml distilled water in a standard flask and store in refrigerator.

EXTRACTION OF AMYLASE

Extract 1g of sample material with 5-10 volume of ice cold 10mM calcium chloride solution overnight at 4^0 C for 3 hours at room temperature. Centrifuge the extract at 54,000g at 4^0 C for 20 minutes. The extract supernatant used as enzyme source.

PROCEDURE

1. Pipette out 1ml of starch solution and 1ml of properly diluted enzyme in a test tube.
2. Incubate at 270 C for 15 minutes.
3. Stop the reaction by the addition of 2ml DNS reagent.
4. Heat the solution in boiling water bath for 5 minutes.

5. While the tubes are warm, add 1ml of potassium tartarate solution
6. Then cool it in running tap water.
7. Makeup the volume to 10ml in addition of 6ml water.
8. Read the absorbance at 560nm.
9. Prepare a standard graph with the concentartion of maltose in X-axis and optical density at Y-axis.

TABULATION

S.No.	Solution		Volume of starch (ml)	Incubate at 27^0C for 15 minutes	Volume of DNB reagent (ml)	Keep in boiling water bath for 5 minutes	Volume of Potassium Sodium tartarate	Optical Density at 560 nm
	Volume (ml)	Concentration (µg)						
Blank	-	-	1.0		2.0		1.0 ml + made upto 10 ml with water	
Standard S1	0.2	20						
S2	0.4	40						
S3	0.6	60						
S4	0.8	80						
S5	1.0	100						
Sample U1	1.0	-						
U2	1.0	-						

CALCULATION

The OD reading ____ corresponds to ____ µg of amylase.

Therefore, 1.0 ml of sample contains = ____µg of amylase.

100 ml of sample contains = ___ x 100/1x1000 mg of amylase.

RESULT

- The amount of amylase present in 1g of sample is _____ gram.

Ex. No. 15

ASSAY OF ANTIBACTERIAL ACTIVITY OF MEDICINAL PLANT BY DISC OR WELL DIFFUSION METHOD

AIM

To determine the antibiotic sensitivity of bacterial strains and inhibitory concentration of antibiotics to bacterial strains.

MATERIALS REQUIRED

Metabolic disc, medicinal plant extract, nutrient agar, bacterial culture, petriplates.

PROCEDURE

1. Select and label the culture that are to be used for antibiotic sensitivity assay
2. Prepare nutrient agar plates
3. Aseptically take a swap of a test culture and inoculates surface of a nutrient agar plate.
4. Allow at least 5 minutes, so the agar surface to dry before.
5. Take forceps and sterilize the tip by dipping in alcohol and flame it. Allow to cool.
6. Carefully take the metabolic disc and place over the agar plate. Gently press the disc to give a
7. better contrast with the agar.
8. Place at least 4 metabolite disc at the same distance apart from each other in the agar plate.
9. Incubate the plate in their in inverted position for 16 to 18 hours at room temperature.
10. Observe the zone of inhibition around the anti-biotic disc.
11. Indicate whether test organism is resistant (no zone of inhibition) or sensitive (clear zone of inhibition) of the metabolite.

RESULT

Microorganisms are _____to plant material.

Ex. No. 16

ASSAY OF ANTIFUNGAL ACTIVITY OF SELECTED MEDICINAL PLANT BY DISC DIFFUSION METHOD

AIM

To determine the antifungal activity using medicinal plant by disc-diffusion method.

MATERIALS REQUIRED

- Medicinal plant extract
- Nutrient agar
- Fungal culture
- Petriplate

PROCEDURE

Preparation of culture medium and inoculation:

Petriplates and agar medium were sterilized for 20 minutes at 1200 C. The rest of the procedure was carried out in laminar air flow. Approx. 20 ml of the media was poured into the sterile petriplate and allow to get solidify. The organism were swabbed using cotton swab.

Disc Diffusion method:

Antifungal activity of the plant extracts were tested using the disc diffusion method. Sterile agar plates were prepared. Aseptically take a swab of a test culture and inoculate on the surface of the agar plates completely, So as to make a lawn. Allow at least 5 minutes so that the agar surface to dry before applying. Take a forceps and sterile the tip by dipping in the alcohol and flame it.

Allow to cool carefully. Take the metabolite disc and place carefully, pass the disc and give a better content with the agar. Incubate the plate at inverse position for 16-18 hours at room temperature. Observe the zone of inhibition around the metabolite disc.

RESULT

Organism was _____ to medicinal plant extract.

Ex. No. 17

PREPARATION OF PLANT TISSUE CULTURE MEDIA
MEDIA PREPARATION

AIM

To prepare or initiate callus culture.

PRINCIPLE

The growth of plant organs is dependent upon the medium used.

MACRO NUTRIENTS

The inorganic constituents present in the medium is necessary for the normal metabolic activity of the plant in culture.

IRON

It is required for the maintenance of cell culture.

NITROGEN

Nitrogen is in the form of Ammonium nitrate.

CARBOHYDRATES

The carbon source of plant is met by adding sucrose in the medium.

GROWTH REQULATING SUBSTANCES

2,4 Dichlorophenoxy acetic acid (2, 4 ,D) enhance the growth rate.

AGAROSE

It provides the semisolid matrix for plants to absorb the material from the medium.

COCONUT WATER

It is recommended for successful growth of plant and it contains growth promoter substance.

VITAMINS

Vitamins B and glycine promote growth.

KINETIN

It enhance the growth of tissue.

GIBBERELLIC ACID

It stimulates the growth.

MATERIAL REQUIRED

Double distilled water, tender coconut, conical flask, aluminium foil.

PROCEDURE

STEP 1: Preparation of stock solution:

It is essential to prepare the stock solution from major salts, miner salts, potassium iodide, iron and vitamin separately. Usually the stocks are prepared in 10X or 100X. The chemicals were dissolved in double glass distilled water contained in reagent bottle or volumetric flask. The stocks were prepared as follows:

Stock A: Major salts (10X) g/l:

- KNO_3 :19
- NH_4No_3 : 16.50
- $MgSo_4.7HO_4$: 3.7
- K_2HPo_4 : 1.70

Stock B: Minor salts (100X) MG/100ml

- H_3BO_3 : 0.620 g
- $Na_2MOO_4.\ 2H_2O$:0.625 g
- $Cocl_2.H_2O$: 0.003 g
- $MnSo_4$: 2.230 g

Stock C: KI (100X) mg/100 ml

Dissolve 83 mg of KI in 100ml of distilled water and stored in amber coloured bottle.

Stock D: Iron EDTA solution (10X) in 1 litre:

1. Dissolved 5.37g of $FeSo_4.2H_20$ in 340 ml distilled water and heated to dissolve if needed.
2. Dissolved 3.725 g of Na_2 EDTA in 250 ml water and heated if needed.
3. Mixed both solutions and made up the volume to 1 litre.

Stock E: Vitamins (100X) mg/100ml

- Nicotinic acid – 50
- Thiamine HCl -- 10
- Glycine – 200

- Pyridoxine HCl- 50
- Myoinositol- 10,000

Stock F: Hormones

IAA
NAA
G3

} 100mg + 2.0 ml of 1 N NaoH. This is made to 100 ml= 1ml

Step 2: Mixing of Medium:

1. Take 400 ml of distilled water in a 2 litre conical flask and added stock solution as follows.
2. Major salts stock/ 100ml.
3. Minor salts stock/ 100ml
4. KI stock/ 1 ml.
5. Fe EDTA stock / 5ml
6. Vitamins stock/ 10 ml
7. Sucrose – 30 g
8. Made up the volume to 1 litre by adding glass distilled water. Adjusted the pH to 5.8 with 0.5 N HCl or 0.2 N KOH.
9. Add 6g of Bacto agar.
10. Add 10 ml of hormone.
11. The flask was shaken well. The medium was kept over a water bath and heated to dissolve. Magnetic stirrer was used for thorough mixing of the chemicals and maintained at warm condition to avoid solidification of the medium. The medium was dispensed into testubes or flask and plugged with non- absorbent cotton wool.

Step 3: Sterilisation of the medium:

The medium was kept in an autoclave and sterilised at 121°C (1.06 kg/cm^2) for 20 minutes. The autoclave and medium was kept at room temperature for cooling. Stants of agar medium were made if necessary before solidification stored the medium at 10°C.

OBSERVATION AND RESULT:

The medium for plant tissue culture callus initiation was prepared and ready for use.

Ex. No. 18

SURFACE STERILIZATION

AIM

To sterilise the room, bench, the plant material, instruments etc.

PRINCIPLE

The prevent the microbial contamination, sterilisation is necessary.

- Heat in the form of stem under pressure is the most practical and most dependable agents for sterilisation.
- Dry heat sterilisation is recommended for glasswares.
- 70% ethanol is most suitable for cleaning beaches, operators bands etc
- 2% thymol in alcohol is the best fumigating agent.
- $KMno_4$ put in formaldehyde is the best and simple form.
- Teepol is the best disinfecting agent for seeds and plants.
- 10% sodium hydrochloride wash is best suited for plants material.

MATERIALS REQUIRED

- 70% alcohol
- 2% thymol in alcohol
- $KMno_4$
- Formaldehyde
- 10% sodium hydroxide
- 10% teepol

PROCEDURE

1. The glasswares were packed and autoclaved for 20 minutes at 15 lbs / inch square pressure.
2. Room Sterilisation:

   The culture room was fumigated by adding 40 g of $KMno_4$ in 100ml of formaldehyde. The heater was left in the room overnight. Ultraviolet were kept on for half an hour.

3. Inoculation room sterilisation:

   For carrying aseptic operation the working surface of the inoculating chamber was surface sterilised by rubbing with 70% alcohol.

4. Operator hands:

   The hands of the operator was thoroughly washed with dettol soap and then wiped with 70% alcohol.

5. Forceps, Blades etc

   The forceps, other cutting instruments were dipped in alcohol and flamed till the fire went off.

6. Plant material:

   The plant parts were washed with tap water and cut into pieces outside inoculation chamber and wiped with concentrated teepol solution by swabbing with cotton. The detergent was washed by shaking in 500ml distilled water in a flask. Rinsing was repeated till the detergent was completely removed. The plant materials were rinsed in 70% alcohol for a minute and sterile water was given till the alcohol was removed. 10% sodium hypochlorite was added and shaken vigorously for 20 minutes. Then the plant materials were taken inside the inoculation chamber and sterile water was used to thoroughly remove the hypochlorite. Now the material is ready for inoculation.

Ex. No. 19

INDUCTION OF MERISTEM CULTURE

AIM

To induce meristem tip culture.

PRINCIPLE

Meristem cultures is the in vitro culture of a generally shiny special dome like structure measuring less than 0.1mm in length and only one or two pairs of youngest leaf primordia, most excised from the shoot apex.

BACKGROUND

The excised shoot tip and meristem can be cultured aseptically on agar solidified simple nutrient medium or on paper bridges dipping into liquid medium and under appropriate conditions will grow out directly into a small leafy shoot or multiple shoots. Alternatively, the meristem may form a small callus at its cut base on which a large number of shoot primordia will develop. These shoot primordia grow out into multiple shoots. Once the shoot have been grown directly from the excised shoot tip or meristem, they can be propagated further by nodal cuttings. This process involves separating the shoot into small segment each containing one mode. The axillary bud on each segment will grow out in culture to form a yet another shoot. The excised stem tips of orchids in culture proliferate to form callus from which some organised juvenile structures known as protocorm develop. When the protocorm are separated and cultured on fresh medium, they develop into normal plants. The stem tips of Cuscuta reflexa in culture can be induced to flower when they are maintained in the dark. Exogenously supplied cytokinins in the nutrient medium plays a major role for the development of a leaf shoot or multiple shoots from the meristem or shoot tip. Generally high cytokinins and low auxin are used in combination for the culture of shoot tip of meristem. Addition of adenine suifate in the nutrient medium also induces shoot tip multiplication in some areas. BAP is the most effective cytokinins commonly used in shoot tip or meristem culture. Similarly, NAA is most effective auxins used in shoot tip culture. Coconut milk and gibberlic acid are also equally effective for the growth of shoot apices in some cases.

MATERIALS REQUIRED

Sodium hypochlorite solution, Sterile MS media containing Vitamin B5, Explants, Jeweler's forceps, surface sterilant, sterile scalpels, sterile petriplates, Binocular dissecting microscope, Laminar Air Flow chamber, 3% Sucrose and 0.8% agar.

PROCEDURE

1. Remove the young twings from the healthy plant. Cut the tip portion of the twig.
2. Surface sterilize the shoot apices by incubation in a sodium hypochlorite solution (1% available chlorine) for 10 minutes. The explants are thoroughly rinsed 4 times in sterile distilled water.
3. Transfer each explant to a sterilize petridish.
4. Remove the outer leaves from each shoot apices with pair of jeweler's forceps. This lessens the possibility of cutting into the softer underlying tissues.
5. After the removal of all the outer leaves, the apex is exposed. Cut off the ultimate apex with the help of scalpel and transfer only those less than 1 mm in length to the surface of the agar medium or to the surface of Filter Paper Bridge. Flame the neck of culture tube before and after the transfer of excised tips. Binocular dissecting microscope can be used for cutting the true meristem or shoot tip perfectly.
6. Incubate the culture under 16 hrs light at 25^0C.
7. As soon as the growing single leafy shoot or multiple shoots obtained from single shoot tip or meristem, transfer them to hormone free medium to develop roots.
8. The plants form by this way are later transferred to pots containing compost and kept under green house condition for hardening.

Ex. No. 20

CALLUS INDUCTION

AIM

To initiate plant tissue culture in Daucus carota or Salonum.

PRINCIPLE

Objects best sterilised for callus initiation are the explants with mature cells cut from the tap root of carrot (Daucus carota) or leaves of tomato plant.

METERIAL REQUIRED

1. Leaves of tomato plant or taproot of carrot.
2. Culture medium.
3. Laminar flow chamber, burner, ethanol etc.
4. Surglass (sterilised)
5. Petriplates, flasks etc (sterilised)
6. Double distilled water (sterilised) in flasks.

PROCEDURE AND CALLUS INDUCTION

Preparation of Medium:

Prepared MS medium was supplemented with 3% sucrose, 1% agar, 2 Mg/1 of IAA and 2 mg/l Kinetin.

Preparation of plant material:

Immature leaves from tomato plant or tap root by carrot were collected and washed with double distilled water in a flask (carrot should be cut into desired size to accommodate the flask). The flask containing the plant organ was placed inside an aseptic laminar flow chamber. The organs were transferred to a sterile petriplate containing double distilled water and cut into uniform pieces approximately 1 cm with the help of the sterile scalper. The pieces were immersed in a flask with 0.1% mercuric chloride for 90 seconds with gentle shaking, pieces were transferred aseptically to a flask containing sterile double distilled water and rinsed thoroughly to remove mercuric chloride. This may be repeated twice or thrice.

Inoculation and incubation:

The edges of the organ pieces were trimmed to get an explants size of about 1 mm thickness. The explants were transferred aspetically on the surface of the agar medium. The mouth of the tube plugged with cotton. The tubes were incubated in a sterile culture room for 2-3 weeks for callus initiation.

RESULTS:

About 10-20 days were required for callus initiation. The response of the leaf disc explants or carrot piece explants to the initiation of callus were seen by increase in the surface area of the explants.

Ex. No. 21

REGENERATION OF SHOOT AND ROOT FROM CALLUS CULTURE

AIM

To regenerate shootlet and from callus culture of Daucus carota.

PRINICLPLE

By adding kinetin and naphthalene acetic acid, the callus can be induced for shoot and root formation.

MATERIAL REQUIRED

1. Sections of callus culture
2. Culture medium.
3. Laminar flow
4. Surgical etc.
5. Petriplates, flasks etc (sterilised)
6. Double distilled water (sterilised) in flasks.

PROCEDURE

Preparation of medium:

MS regeneration medium was prepared with 3% sucrose, 1 mg/l naphthalene acetic acid and 1 mg/ l kinetin. Agar (0.9%) was used to solidify the medium.

Preparation of plant materials:

About 2 to 5 mm diameter of calli was picked with a sterile flattened needle and transferred into a conical flask containing solid MS regeneration medium for plant regeneration. Incubated the culture under continuous light at 25± 1 ˚C and observed for a period of 2-3 weeks for the growth of the fairy green plants and white roots from calli.

RESULT

Kinetin and naphthalene acetic acid induced the development of shoot and root system from callus portions.

KARPAGAM ACADEMY OF HIGHER EDUCATION
(Deemed to be University Established Under Section 3 of UGC Act 1956)
Pollachi Main Road, Eachanari Post, Coimbatore - 641 021. INDIA
Phone : 0422-6471113-5, 6453777 Fax No : 0422 -2980022-3
Email : info@karpagam.com Web : www.kahedu.edu.in

DEPARTMENT OF BIOCHEMISTRY

SUBJECT NAME: PRACTICAL II - PLANT BIOCHEMISTRY AND MICROBIOLOGY

SUB.CODE: 17BCP112

SEMESTER: I **CLASS: I M.Sc., BIOCHEMISTRY**

Possible Viva Questions

1. Differentiate Quantitative and Qualitative assay.
2. What is principle behind Salkowski's test?
3. What are the carbohydrate tests employed for qualitative analysis of plant extract?
4. What is the principle of estimation of tannin?
5. What is the principle of estimation of flavonoids?
6. What is the principle of estimation of phenol?
7. How will you estimate chlorophyll from given plant extract?
8. What is asepsis and aseptic condition?
9. Define fumigation.
10. How do you create aseptic technique in laminar flow?
11. What is serial dilution?
12. Why colour and nature of colony of different bacteria vary?
13. Give countable colony range of bacteria.
14. How do you select dilution for the specific microorganism?
15. Why is pure culture needed?
16. Differentiate pour, spread and streak plate technique.
17. What is a bacterial colony?
18. What is the purpose for spread plate technique?
19. Why it is important to invert the petri plates during incubation?
20. Why is smear needed for staining?
21. What are the methods available to fix the smear?

22. Differentiate dye and stain.
23. What are the kind of dyes used to stain the cell wall of bacteria?
24. Differentiate gram positive and gram negative cell wall.
25. Why is gram stain called differential stain?
26. Which part of the bacterial cell wall involved mostly with Gram staining, and why?
27. Give examples of endospore forming bacteria.
28. Why are endospores difficult to stain?
29. What do endospore stains have in common with acid fast stain?
30. What is the principle of indole test?
31. Which is the major ingredient in indole test medium?
32. Why is methyl red test used methyl red indicator?
33. Why is phenol or phenolphthalein not used in methyl red test?
34. How is mixed acid fermentation of bacteria detected?
35. What are the reagents used in Voges Proskauer test?
36. Name the triple indicator used in citrate utilization test.
37. What are the principle component in citrate medium?
38. How do you interpretate catalase test result?
39. What is the difference between %T and absorbance?
40. How do you prepare series of dilutions to get final dilution of 10^{-10}?
41. Define biomass.
42. Which microbes grew best in the acid, alkaline and neutral pH range?
43. Why are buffers added to culture media?
44. How does thermopiles withstand high temperature?
45. What are the limitations for using boiling water as a means of sterilizing materials?
46. Name any two vitamins used in tissue culture medium?
47. How do you sterilize tissue culture medium?
48. Mention the role of light and dark in explant incubation.
49. What are the uses of protoplast fusion?
50. Why are cytokinins added in tissue culture medium?

KARPAGAM ACADEMY OF HIGHER EDUCATION
(Deemed to be University Established Under Section 3 of UGC Act 1956)
Pollachi Main Road, Eachanari Post, Coimbatore - 641 021. INDIA
Phone : 0422-6471113-5, 6453777 Fax No : 0422 -2980022-3
Email : info@karpagam.com Web : www.kahedu.edu.in

DEPARTMENT OF BIOCHEMISTRY

SYLLABUS

SUBJECT NAME: PRACTICAL II - PLANT BIOCHEMISTRY AND MICROBIOLOGY

SUB.CODE: 17BCP112

SEMESTER: I **CLASS: I M.Sc., BIOCHEMISTRY**

Programme objective
To have impact knowledge on plant biochemistry related screening techniques and microbial technology, due to its higher applications in research field.

Programme learning outcome
Students who have learned about phytochemical screening with tissue culture techniques gained knowledge on how to startup research with plant biology. Also, microbial culture techniques made them to have well expertise in microbiology research field.

PLANT BIOCHEMISTRY

1. Phytochemical screening of any one selected medicinal plant
2. Estimation of Tannins
3. Estimation of Flavonoids
4. Estimation of Chlorophyll
5. Estimation of Phenols

MICROBIOLOGY

6. Isolation of pure culture – serial dilution, pour plate, spread plate, streak plate methods.
7. Colony morphology – colony counting.
8. Staining techniques- simple, differential, spore, and fungal staining.
9. Antibiotic resistance / sensitivity test (Disc method)
10. Estimation of bacteria- growth curve of bacteria and generation time.
11. Identification of microorganisms – biochemical tests (IMVIC test)(Group Experiment)
12. Microbiology of potable water
13. Isolation, characterization and purification of ANY one of the following microbial enzymes
    a) Amylase

b) Protease

14. Assay of Antibacterial of ANY ONE selected medicinal plant by Disc or Well diffusion and broth dilution method.
15. Assay of antifungal activity of ANY ONE selected medicinal plant by Disc or Well diffusion. TLC- Bioautography.

PLANT TISSUE CULTURE (Group experiment)

16. Preparation of tissue culture media
17. Surface sterilization
18. Induction of meristem culture
19. Callus induction.
20. Regeneration of shoot and root from callus culture.

REFERENCES

1. Sadasivam, S.,and Manickam, A., (2009). Biochemical Methods, New Age, International Publishers, New Delhi.
2. Rajan S., and Selvi C., (2011). Experimental Procedures in Life Sciences. Anjana Book House, Chennai.