UPSTREAM PROCESSING TECHNOLOGY LABORATORY MANUAL

VII Semester (10BTL77)

Name of the Student :

Semester /Section :

USN :

Batch :

DAYANANDA SAGAR COLLEGE OF ENGINEERING

Accredited by National Assessment & Accreditation Council (NAAC) with 'A' Grade

(An Autonomous Institution affiliated to Visvesvaraya Technological University, Belagavi
&
ISO 9001:2008 Certified)

DEPARTMENT OF BIOTECHNOLOGY

SHAVIGE MALLESWARA HILLS , KUMARASWAMY LAYOUT

BENGALURU-560078

Vision of the Institute

To impart quality technical education with a focus on Research and Innovation emphasising on Development of Sustainable and Inclusive Technology for the benefit of society.

Mission of the Institute

- To provide an environment that enhances creativity and Innovation in pursuit of Excellence.

- To nurture teamwork in order to transform individuals as responsible leaders and entrepreneurs.

- To train the students to the changing technical scenario and make them to understand the importance of Sustainable and Inclusive technologies.

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DEPARTMENT OF BIOTECHNOLOGY, BENGALURU-560078

VISION OF THE DEPARTMENT

To impart quality education, training and research in multidisciplinary domains of Biotechnology for the benefit of society and environment.

MISSION OF THE DEPARTMENT

- **To provide globally acceptable technical education in the field of biotechnology by encouraging innovative thinking with practical insights.**
- **To promote team work and nurture students to serve society with ethical and environmental responsibilities.**
- **To foster students for higher studies, R&D activities and professional career in emerging trends of biotechnology.**

PROGRAMME EDUCATIONAL OBJECTIVES [PEOs]

PEO-1: Skill -Enable our graduates to identify, analyse and solve industrial and environmental problems by implementing acquired skills.
PEO-2: Career - Encourage our graduates to apply their engineering knowledge as an individual or in a team to excel in higher studies, research, teaching and industry.
PEO-3: Lifelong learning - Instil in our graduates a desire to engage in lifelong learning that will foster their career with an impact on society.
PEO-4: Ethics - Inculcate in our graduates to develop high level of professionalism and ethical attitude with awareness of current issues in relation to safety, health and environment.

PROGRAMME SPECIFIC OUTCOMES [PSOs]

Graduates will be able to
PSO-1: Apply engineering principles to biological systems.
PSO-2: Analyze the genome and proteome by advanced molecular techniques and computational tools to address major challenges in Pharma and Health.
PSO-3: Develop eco-friendly solutions to address complex environmental problems.

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UPSTREAM PROCESSING TECHNOLOGY LABORATORY (SYLLABUS)

VII SEMESTER B. E. (BT)

Sub. Code:10BTL77 | **IA Marks :25**
Hrs/Week : 03 | **Exam Hrs :03 Hrs**
Total Hrs:09/week | **Exam Marks :50**

Course Objectives:

1. To gain knowledge of media preparation techniques.
2. To learn different plant tissue culture techniques.
3. To understand the techniques of secondary metabolites isolation.
4. To learn lycopene isolation method.
5. To learn citric acid isolation from *Aspergillus niger*.

Syllabus:

Sl. No	Experiments
1.	Preparation of media for plant tissue culture
2.	Callus Induction Techniques – Carrot/Beet root/ or any other material
3.	Development of suspension culture from callus
4.	Induction of Secondary metabolite – Anthocyanin/cateranthin
5.	Estimation of Lycopene from tomato fruits
6.	Estimation of DNA (by DPA method)
7.	Protein estimation by Lowry’s method/Brandford’s method
8.	Development of inocula; lag time effect
9.	Shake flask studies; Comparison of biomass yield in defined and complex media
10.	Production and estimation of citric acid from *Aspergillus niger*

Course Outcomes:

1. Students will gain knowledge on media preparation.
2. Students will learn how to secondary metabolites induction.
3. Students will learn callus and suspension culture techniques.
4. Students will isolate and understand the role of lycopene.
5. Students will estimate the DNA and protein from different sources.
6. Students will learn importance of lag phase.
7. Students will understand the role of *Aspergillus niger* in citric acid production.

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BENGALURU – 560078

DO's

- Students should come before the commencement of laboratory (Upstreatm Processing Technology, CD Sagar III floor).
- Students should follow lab coats is mandatory.
- Bags, and other personal items must be stored in designated place.
- Students should read the instructions for safe and disposal of materials.
- Come prepare with the viva, procedure, and other details of the experiment.
- Secure long hair, loose clothing & know safety and emergency procedures.
- Do check for the correct ranges/rating and carry one meter/instrument at a time
- Inspect all equipment/meters for damage prior to use
- Conduct the experiments accurately as directed by the teacher.
- Immediately report any sparks/ accidents/ injuries/ any other untoward incident o the faculty /instructor.
- Handle the apparatus/meters/computers gently and with care
- In case of an emergency or accident, follow the safety procedure.
- Switch OFF the power supply after completion of experiment

DONT's

- The use of mobile/ any other personal electronic gadgets is prohibited in the laboratory.
- Do not make noise in the Laboratory & do not sit on experiment table.
- Do not make loose connections and avoid overlapping of wires
- Don't switch on power supply without prior permission from the concerned staff.
- Never point/touch the CRO/Monitor screen with the tip of the open pen/pencil/any other sharp object.
- Never leave the experiments while in progress.
- Do not insert/use pen drive/any other storage devices into the CPU.
- Do not leave the Laboratory without the signature of the concerned staff in observation book

Experiment No: 01 Date:

PREPARATION OF PLANT TISSUE CULTURE MEDIUM

Aim: To prepare Murashige and Skoog (MS) media for plant tissue culture.

Introduction: Murashige and Skoog medium or (MSO or MS0 (MS-zero)) is a most commonly used plant growth medium in the laboratories for cultivation of plant cell culture. MSO was invented by plant scientists Toshio Murashige and Folke K. Skoog in 1962 during Murashige's search for a new plant growth regulator.

The culture medium is one of the most important components of plant cell and tissue culture method. The successful application of plant culture medium with its right composition in addition to its components is one of the most important pre-requisite of a plant tissue culture experiment. Another important function of the culture medium is to provide the right physical environment for cells and tissues to grow on solid medium. The media also performs a function like soil by providing a physical support matrix. All plant cells require water, nutrients and plant growth regulators.

The requirements of plant cell culture may be subdivided into 3 groups:

1. Inorganic nutrients
2. Organic nutrients
3. Plant growth regulators

Inorganic nutrients: Inorganic nutrients are mineral elements and based on their essential concentration they are classified into 2 groups: macroelements, present in large supplies (mM concentration) and microelements as trace elements.

Macroelements:

- Nitrogen: supplied in the form of nitrate ions and ammonium ions (KNO_3 and NH_4NO_3). Nitrogen is a component of protein, nucleic acid, chlorophyll, some coenzymes and is required in great amounts.
- Sulphur: supplied in the form of sulphate ions. Sulphur is a vital component of amino acids, vitamins, cofactors, iron sulphur protein (electron carrier).
- Phosphorous: supplied as a phosphate ion (PO4-). Added as Na/K dihydrogen phosphate. It is an essential component of nucleic acid, phospholipids and in energy rich compounds.
- Cations: supplied as KNO3 salt. Potassium functions as osmoregulators, cation-anion balance, and pH stabilization.

Microelements:

- Iron: added as iron sulphate [Fe (III)]. EDTA is usually used in conjunction with iron sulphate. This complex with iron so as to allow slow and continuous release of iron into the medium.Uncomplexed iron usually precipitates out of the medium as ferric oxide. It is required by the plant for chlorophyll synthesis as a constituent of cytochrome and Fe-S protein.

- Boron: added as boric acid. It is a component of cell wall required for cell division of optical meristem.
- Cobalt: added as cobalt chloride. It is a component of some vitamins.
- Copper: added as copper sulphate. It as serves as an enzyme cofactor and function in electron reactions.
- Iodine: improves growth of roots and callus, added in the form of KI.
- Manganese – Added in the form of $MnSO_4$. Manganese is found in metalloproteins.
- Molybdenum – Added as sodium Molybdate. It is an enzyme cofactor.
- Zinc – Added as $ZnSO_4$ required for activity of various enzymes.

Organic Nutrients:

While green plants are autotrophic, most culture systems in early stage is heterotrophic. Plant tissue culture system requires an organic carbon and energy source and complex organic molecule such as vitamins and cofactors for healthy growth.

- Carbon Source: Sucrose is most commonly used sugar in plant tissue culture media, but glucose, fructose and sorbitol are used in some formulations.
- Vitamins and Co-factors: two vitamins, thiamine (B1) and myoinositol(Vit B) are essential for plant tissue culture. Other vitamins such as biotin, pantothenic acid (niacin), pyridoxine (Vit B6), folic acid, ascorbic acid (Vit C) and tocopheryl acetate (Vit E) are added to the media.
- Complex Organic Supplements: complex additions such as banana powder and liquid endosperm of coconut are frequently used in plant tissue culture media such as kinetin, benzyl aminopurine and zeatin media to improve growth.

Plant Growth Regulators:

There are 5 main classes of plant growth regulators.

- Auxins: they promote both cell division and cell growth. Auxins are synthesized in stems and root spaces and transport along the plant axis. Auxins commonly used in plant tissue culture are 2, 4-dichlorophenoxy acetic acid (2, 4-D), Indole 3-acetic acid (IAA), Indole-3-butyric acid (IBA), 1-napthalene acetic acid (NAA). Auxins together with cytokines are used in plant tissue culture to control differentiation and morphogenesis.
- Cytokinins: they promote cell division, growth and development. Naturally occurring cytokinins are purine derivatives. They are frequently used in plant tissue culture media such as kinetin, benzyl aminopurine and zeatin.
- Gibberellins: Major action of gibberillin is stimulation of cell stem elongation and flowering. Gibberillic acids are commonly used in plant tissue cultures (GA, GA4, and GA3).
- Abscissic Acid: Primarily involved in water-stem responses, seed germination and enhances somatic embryogenesis.
- Ethylene: is gaseous and is associated with fruit ripening in climatic fruits.

Support Matrices of Gelling Agents:

In some plant cell and tissue culture system a solid or semi solid matrix is needed to support tissue explants while allowing contact with media. The most commonly used support matrices are formed from gelling agents such as agar, agarose, gelatin, gelatin gums.

Agar is the most commonly used gel in plant tissue culture. It is a complex polysaccharide extracted from red algae (Rhodophyceae) and comprises 2 fractions-agar (70%) & agar pectic (30%). Agarose in the jelly fraction and consists of a polymer of D-galactose and 3, 6-anhydrogalactose monosaccharide units. Agropectin is the non gelling fraction and consists of polymer of sulphur.

The required basal medium can be prepared by either dissolving the commercially available basal salt mix on by preparing the mix from individual ingredients. An alternative is to prepare macronutrients & micronutrients stock solution and store these at 40 C. Until used, organic compounds should not be stored in solution for more than 2 weeks. Heat labile compounds such as plant growth regulator, antibiotics are added to the medium after autoclaving.

Materials and Requirements:

- Distilled water, sucrose, gelling agent, 1M NaOH or 1M HCl to adjust the pH, micronutrients stock, macronutrients stock, organic compound stock.
- Equipment – Balance, pH meter, autoclave, LAF cabinet.
- Glassware – conical flask, phyta jars, beakers, pipettes.

Procedure:

- Add approximately half the volume of water to a conical flask
- Weigh out the appropriate amount of basal salts (4.4g) or measure out appropriate volume of stock solution .Add the water and 30 g sucrose
- When all solids have dissolved , adjust the pH 5.7 using 0.1M NaOH or HCl
- Make up the volume to 1 litre using distilled water
- Weigh 10 g agar and add to the above medium
- Melt the agar by heating
- Divide the medium into tissue culture bottles and close the bottles with screw caps
- Sterilize the culture bottles by autoclaving at 1210 C for 15 min 15 psi
- After autoclaving, ensure the caps are immediately lightened.

Preparation of hormone stocks:

- Weigh out 0.3g of IAA
- Sterilize the IAA by placing under UV light for 30 mins. Transfer to a sterile volumetric flask.
- Dissolve the IAA in few drops of ethanol and make up the volume to 100 ml using sterile distilled water.
- Weigh out 0.1mg of kinetin and sterilize under UV light
- Transfer to a flask and dissolve in 100 ml of sterile distilled water
- Add 30 ml of IAA stock and 1.5 ml of kinetin stock to each bottle aseptically. Mix well and allow the contents to solidify and use immediately or store at 40C .

Murashige and Skoog (MS) media composition:

Macroelements	**g**
NH_4NO_3	1.65
KNO_3	1.90
$CaCl_2.2H_2O$	0.44
$MgSO_4.7H_2O$	0.37
KH_2PO_4	0.17
Microelements	**mg**
$FeSO_4.7H_2O$	27.80
$Na_2EDTA.2H_2O$	33.60
KI	0.83
H_3BO_4	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Organic supplements	**mg**
Myoinositol	100
Nicotinic acid	0.05
Pyridoxine HCl	0.05
Thiamine HCl	0.05
Glycine	0.20
Sucrose	30g
Agar	10g

Viva-Voce

- What is media?
- What are the macroelements in tissue culture?
- What is the role of microelements in plant development?
- Explain the importance of growth hormones?
- Difference between simple and complex media?

Experiment No: 02 Date:

CALLUS INDUCTION TECHNIQUES

Aim: To induce callus from root explant of ***Daucus carota***

Introduction: As a first step in many tissue culture experiments it is necessary to induce mcallus formation from the primary explant. The explant may be an aseptically germinated seedling or surface sterilized roots, stems, leaves or reproductive structures.Callus is wound tissue produced in response to injury.Not all o thr cells in an explant contribute to the formation of callus and more importantly, certain callus cell types are competent to regenerate organized structures, but other cell types do not appear to be competent to express totipotency.Callus formation is controlled by the level of plant growth regulators in the culture media. Concentrations of the plant growth regulators can vary for each plant species and can even depend on the source of explant or individual plant.
Culture conditions like temperature and light are also important in callus formation and development. Once established, callus cultures may be used for the study of protoplast isolation, cell types, cellular selection, somatic embryogenesis, organogenesis and secondary product formation.

Requirements
Explant material, disinfectants, callus induction medium, Laminer Air Flow (LAF), culture room,and glassware.

Procedure

- Root explant from a young ***Daucus carota*** are collected.
- The explant are trimmed to about 1.5-2.0cm each with a sterile blade. As a first step of sterilization the explant are washed with a fungicide bavistin and 10%soap solution for 10 min.
- The explant are washed under running tap water for 30 min.
- Surface sterilization is done with 0.1%mercuric chloride for 7 min.
- To remove traces of mercuric chloride the explant are washed with distilled water 2-3 times.

Dressing of the tissue:

- It is carried out in the LAF. The ends are cut to eliminate the deleterious effect that would be caused by the absorption of disinfectants.
- Since callus induction is a wound response, a longitudinal cut is made to the root explant and is inoculated in an inverted position in the medium.
- The following concentrations of hormones may be tried out.
  2,4-dichlorophenoxy acetic acid- 2.5mg/l, NAA-0.5mg/l, 2,4 D+NAA- 0.5mg each per litre.
- The cultures are incubated at 25deg C with 16 hrs light and 18 hrs dark (photoperiod) in the tissue culture chamber room for callus induction.
- Subculturing of about 0.3 to 0.5g callus is carried out from time to time and observed for morphological and other growth changes.

Observation and Result

Composition of Murashige and Skoog Medium

Macroelements	**g**	**Microelements**	**mg**
NH_4NO_3	1.65	$FeSO_4.7H_2O$	27.80
KNO_3	1.90	$Na_2EDTA.2H_2O$	33.60
$CaCl_2.2H_2O$	0.44	KI	0.83
$MgSO_4.7H_2O$	0.37	H_3BO_4	6.20
KH_2PO_4	0.17	$MnSO_4.4H_2O$	22.30
		$ZnSO_4.7H_2O$	8.60
		$Na_2MoO_4.2H_2O$	0.25
		$CuSO_4.5H_2O$	0.025
		$CoCl_2.6H_2O$	0.025

Organic supplements	**mg**
Myoinositol	100
Nicotinic acid	0.05
Pyridoxine HCl	0.05
Thiamine HCl	0.05
Glycine	0.20
Sucrose	30g
Agar	10g

Viva –Voce

1. What is callus?
2. Which part from plant do you select for the production of callus?
3. What is the medium used for the callus induction?
4. What are the growth factors that should be added to the callus medium?
5. How do you prepare MS medium?

Experiment No: 03 Date:

DEVELOPMENT OF SUSPENSION CULTURES FROM CALLUS

Aim: To develop suspension cultures from ***Daucus carota***

Introduction: Tissues and cells cultured in a liquid produce suspension cultures of single cells and cell clumps. These are called suspension cultures. Liquid culture must be constantly agitated on a gyratory shaker at 100 to 250 rpm to facilitate aeration and dissociation of cell clumps into smaller pieces. In suspension cultures cells grow faster than callus cultures and hence need sub culturing every week. They allow more accurate determination of the nutritional requirement of cells and are the only system amenable to scaling up for a large scale production of cell products and even somatic embryos.
Initiation of suspension cultures is important for the generation of cell lines, secondary metabolite production and separation of somatic embryos.

Requirements
Well developed friable callus, suspension culture medium, laminar air flow chamber, rotary shaker, culture room.

Procedure

- Collect the callus stocks gently break up pieces of ***Daucus carota*** callus in a sterile Petri dish using forceps.
- Carefully transfer the small pieces of callus to the MS medium using sterile forceps.
- Incubate the flask for one week on gyratory shaker set at 125rpm.
- Sub culture cell suspension every week.
- Pipette out 1-2 ml of suspension into 4 Petri dishes of agar solidified media, spread evenly over the surface of the medium.
- Record the occurrence of contaminants and the time of development of a stabilized finely dispersed cell suspension culture.
- Observe the growth of callus from cell suspension after 4, 6, 8 weeks. Record the number of callus colonies on each Petri dish.

Observation and result

Composition of Liquid Broth

Reagent	Quantity(ml/l)
Stock A	20
Stock B	20
Stock C	5
Stock D	5
Stock E	5
Stock F	5
Thiamine HCL	0.5
Pyridoxine HCl	0.5
Nicotinic acid	0.5
Glycine	2.0
Myoinositol	1000mg
Sucrose	30gm
pH	5.6 -5.8

Viva-Voce

1. What is suspension culture?
2. What is the use of suspension culture?
3. What is the scientific name of carrot?
4. How do you provide aeration to the suspension culture
5. Difference between liquid and solid media?

Experiment No: 04 Date:

ARTIFICIAL SEED PRODUCTION

Aim: To produce synthetic seed from plant parts

Introduction: Artificial seeds were first introduced in 1970 as a novel analogue to plant seeds. The production of artificial seeds is useful for plants which do not produce viable seeds. It represents the method to propagate these plants. Artificial seeds are small sized and so advantageous in handling shipping and planting.
Artificial seeds can be produced by plant propagule in a matrix, which will allow it to grow into a plant. The plant propagules may constitute shoot buds, somatic embryos that have been grown aseptically in tissue culture. In culture these plant propagules can easily grow into individual plants.
The encapsulation matrix is a hydrogel made of natural extracts from sea weeds (agar, carageenam/ alginate), plants (Arabic/tragacamph), seed gums (gar, locust bean gum or tamarind) or microorganisms (dextran, gellan, or xanthangum).These com[pounds will gel when mixed with or dropped into an appropriate electrolyte (copper sulphate, calcium chloride, ammonium chloride)

Principle: Alginate is a straight chain, hydrophilic, colloidal, and composed of hydro-β-D manuronic acid residues and with 1-4 linkages. When it is mixed with calcium, an ion exchange reaction occurs and sodium ions in Sodium alginate are replaced by calcium ions forming calcium alginate. Surface complexing results in gelling of matrix and resulting in bead like structures within 30 min.

Procedure

- Somatic embryos were induced in *Leptadenia reticulata* leaf cultures by sub culturing the callus into embryo induction medium.
- Encapsulation
- Cotyledonary and mature embryoids developed on the induction medium were mixed with 2 % solution of Sodium Alginate prepared using hormone free nutrient medium.
- The solution containing embryoids were dropped into 100 ml of 100mM calcium chloride solution using special plastic pipettes.
- It is allowed to harden 9 hardness can be controlled by controlling concentration of the complexation solution and time)
- Complexation solution was decanted and beads were rinsed in hormone free medium followed by water.
- The encapsulated embryoids were kept for germination.

Observation and results.

Viva-Voce

1. What is embryo?
2. What is use of artificial seeds?
3. Use of sodium alginate?
4. What is the use of encapsulation?
5. Use of leaf culture?

Experiment No: 05 Date:

ESTIMATION OF ANTHOCYANIN CONTENT IN GRAPES BY pH - DIFFERENTIAL SPECTROPHOTOMETRY

Assay Title: Determination of total anthocyanin content in grape extract by pH-Differential Spectrophotometry.

Introduction: This assay can be used to determine total anthocyanin in grape extract. Determination is performed by using a pH differential method at pH 1.0 and pH 4.5. The difference in absorbance at 510 nm is proportional to the anthocyanin content.

Principle: Anthocyanin pigments change hue and intensity with pH. At pH 1.0, anthocyanins exist in the colored oxonium or flavylium form and at pH 4.5 they are predominantly in the colorless carbinol form. An aliquot of an aqueous anthocyanin solution is adjusted to pH 1.0 and another aliquot to pH 4.5. The difference in absorbance is proportional to the anthocyanin content. Determination of anthocyanin content is based on Lambert-Beer's Law. Molar absorbance values for purified pigments taken from literature are used, making it unnecessary to determine them. Since most fruits contain a mixture of the major anthocyanins and since each anthocyanin can have slightly different extinction coefficients, results may vary depending on the standard chosen. Even though delphinidin-3-glucoside is the major anthocyanin in bilberry, the total anthocyanin content is calculated as cyanidin-3-glucoside because of its historical usage for similar assays and its wide commercial availability.

Standard: Direct quantitation against reference standard is not necessary. Literature molar absorbance value for purified cyanidin-3-glucoside in aqueous pH 1 buffer is used instead.

Pigment	MW†	Loge	ε	Solvent	λ
Cyd-3-glu	449.2	4.43	26,900	pH 1 buffer	510 nm

Molecular weight does not include the weight of chloride counter ion or a water molecule of hydration.

Apparatus:

- Calibrated analytical balance accurate to ±0.1 mg
- Pipet, Class A, assorted sizes
- Spectrophotometer
- Volumetric flasks, assorted sizes

Reagents:

- pH 1.0 Buffer

  Dissolve 1.49 g KCl into 100 mL deionized water. Carefully pour 1.7 mL concentrated HCl into 100 mL deionized water for 0.2 N. Mix 25 mL of the KCl solution with 67 mL of the 0.2 N HCl solution. Adjust pH 1.0 ± 0.1 if necessary.

- pH 4.5 Buffer

  Dissolve 1.64 g sodium acetate in 100 mL deionized water. Adjust to pH 4.5 ± 0.1 with HCl.

Sample Preparation:
Accurately measure 50 ml of grape extract into a 100 mL volumetric flask. Adjust the volume to 100 ml using distilled water. Centrifuge the solution at 3,500 rpm for 10 mins at room temperature.
Take 1.0ml of the supernatent and place it into a 25 ml volumetric flask. Dilute to volume with pH 1.0 buffer and mix.
Remove a second 1.0 mL aliquot of the solution (supernatent) and place it into a 25 mL volumetric flask. Dilute to volume with pH 4.5 buffer and mix.

Procedure:
Zero spectrophotometer with distilled water.
Sample turbidity (haze) is corrected for by measuring the absorbance at 700 nm.
Measure the absorbance of the pH 1.0 and pH 4.5 sample preparations at 510 nm.
Notice: Dilute sample further if absorbance is greater than 1.0 AU.

Calculations:
Calculate the difference in absorbance between the two samples using the following equation.
Absorbance = (A_{510nm} pH 1.0 - A_{700nm} pH 1.0) - (A_{510nm} pH 4.5 - A_{700nm} pH 4.5)
Calculate the %w/w of total anthocyanins in the sample.

$$\%w/w = \frac{A}{\varepsilon L} \times MW \times DF \times \frac{V}{Wt} \times 100\%$$

Where:
A = Absorbance
ε = Cyd-3-glu molar absorbance (26,900)
MW = anthocyanin molecular weight (449.2)
DF = dilution factor
V = final volume (mL)
Wt = sample weight (mg)
L = cell pathlength (usually 1 cm)

Observations and Results

Viva-Voce

1. What is the difference between primary metabolites and secondary metabolites?
2. Give some examples for secondary metabolites.
3. What is the medium used for the production of anthocyanins?
4. Give some examples of pigments that are considered as secondary metabolites.
5. Explain Lambert-Beer's Law.

Experiment No: 06 Date:

ESTIMATION OF LYCOPENE FROM TOMATO FRUITS

Lycopene is responsible for the red colour of tomato and the fleshy part of water melon. It is a carotene having the formula C40H56. Though it has no nutritional value,its contribution to the colour of tomato has a great role in consumer acceptability.

PRINCIPLE

The carotenoids in the sample are extracted in acetone and then taken up in petroleum ether. Lycopene has absorption maxima at 473nm and 503nm, one mole of lycopene when dissolved in one liter light petroleum (40-60) and measured in a spectrophotometer at 503 nm in 1cm light path gives an absorbance of 17.2 X 104. Therefore, a concentration of 3.1206ug lycopene /mL gives unit absorbance.

Materials

- Acetone (AR Grade)
- Petroleum ether 40 -60 (AR)
- Anhydrous Sodium Sulphate
- 5 % Sodium Sulphate

Procedure

1. Take 3-4 tomato fruits (Sample) and pulp it well to a smooth consistency in a waring blender.
2. Weigh 5- 10 g of this pulp.
3. Extract the pulp repeatedly with acetone using pestle and mortar or a waring blender until the residue is colourless.
4. Pool the acetone extracts and transfer to a separating funnel containing about 20 ml petroleum ether and mix gently.
5. Add about 20 ml of 5 % sodium sulphate solution and shake the separating funnel gently. Volume of petroleum ether might be reduced during these processes because of its evaporation. So add 20 mL more of petroleum ether to the separating funnel for clear separation of two layers. Most of the colour will be noticed in the upper petroleum ether layer.
6. Separate the two phases and re-extract the lower aqueos phase with additional 20 mL petroleum ether until the aqueous phase is colourless.
7. Pool the petroleum ether extracts and wash once with a little distilled water.
8. Pour the washed petroleum ether extract containing carotenoids in to a brown bottle containing about 10 g anhydrous sodium sulphate. Keep it aside for 30 min or longer.
9. Decant the petroleum ether extract into a 100 mL volumetric flask through a funnel containing cotton wool. Wash sodium sulphate slurry with petroleum ether until it is colourless and transfer the washings to the volumetric Flask /
10. Make up the volume and measure the absorbance in a Spectrophotometer at 503nm using petroleum ether as blank.

Calculation

Absorbance (1 Unit) = 3.120g lycopene / mL

$$\text{mg lycopene in 100g sample} = \frac{31.206 \times \text{Absorbance}}{\text{Wt. of Sample (g)}}$$

Observations and Results

The amount of Lycopene present in the given sample is ———— mg/100g of juice.

Viva-Voce

1. What is the use of petroleum ether in the extraction of lycopene?
2. What is the use of acetone in the estimation of lycopene?
3. What is the chemical formula of lycopene?
4. Which part of the solution in the separating funnel do you take to estimate the lycopene and why?
5. What is the use of lycopene?.

Experiment No: 07 Date:

ESTIMATION OF DNA BY DIPHENYL AMINE METHOD

Aim: To estimate the amount of DNA present in the sample by Diphenyl amine method

Principle:

When DNA is treated with DPA under acid conditions, a blue colored complex is formed with a sharp absorption maximum at 595 nm. This reaction is given by 2-deoxypentoses in general and is not specific for DNA. In acid solution, the straight chain form of a deoxypentose is converted to the highly reactive β- hydroxyl levunaldehyde which reacts with diphenyl amine to give a blue complex. In DNA only the deoxyribose of the purine nucleotides reacts, so that the value obtained represents half of the total deoxyribose present.

Reagents

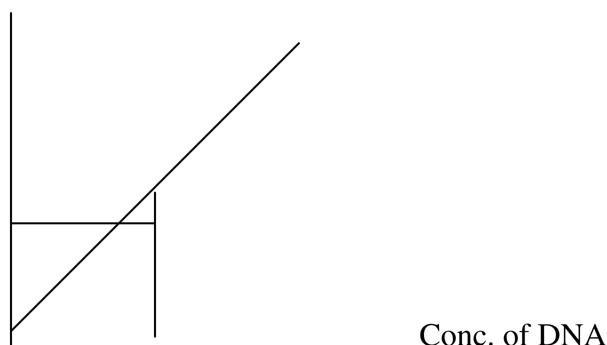
DNA: 10mg is dissolved in 50ml of buffered saline.
Buffered saline: 0.15 m/l Sodium citrate and 0.15M/l NaCl, pH 7.0
Diphenyl amine reagent
Dissolve 10gm of pure DPA in 1 l of glacial acetic acid and add 25ml of conc. H_2SO_4. This solution should be made fresh.

Procedure

- 10mg of DNA is accurately weighed and dissolved in 50ml of buffered saline.
- 7 well cleaned test tubes are taken and marked 1, 2, 3…..7.
- Aliquots of 0.4ml, 0.8ml, 1.2ml, 1.6ml, and 2.0ml of DNA are taken except for blank and test.
- 2 ml of unknown sample is added to the test tube 7.
- The volume is made up to 2 ml in each of the test tubes with buffered saline.
- 4 ml of DPA is added to each tube and the tubes kept in boiling water bath for 10 min.
- The tubes are cooled and the OD read at 595nm.
- A graph of conc. of DNA on the X- axis and OD on the Y- axis is plotted.
- The conc. of unknown DNA is calculated from the graph.

Result: The amount of DNA present in the unknown sample was found to be

Observation



Sl No	Volume of DNA solution (ml)	Volume of buffer (ml)	Volume of DPA (ml)		Conc. Of DNAμgm	OD at 595 nm
1(B)	0.0	2.0	4	Incubate in boiling water bath for 10 min		
2	0.4	1.6				
3	0.8	1.2				
4	1.2	0.8				
5	1.6	0.4				
6	2.0	0.0				
7	2.0(unknown)	0.0				

Viva-Voce

1. Give some examples for nucleic acids?
2. How do you estimate the DNA?
3. Explain the principle of DNA estimation by DPA method?
4. Which compound will react with diphenylamine to give a blue colored complex?
5. Use of acid.

Experiment No: 08 Date:

ESTIMATION OF PROTEIN BY LOWRY'S METHOD

Aim: To estimate the amount of protein in the given sample by Lowry's Method.

Principle : This method is about 10 times more sensitive than the Biuret method. The reagent Folin-Ciocalteaeu reagent is quite complex and contains phospho molybdic acid and tungstate.Protein reacts with the Folin-Ciocalteaeu reagent to give a colored complex. The color so formed is due to the reaction of the alkaline copper with proteins as in the Biuret test and the reduction of the phosphomolybdate by the tyrosine and tryptophan. The intensity of color depends on the amount of the aromatic acids present and thus varies for different proteins.

Reagents:
Alkaline copper reagent
Solution A
2% Sodium carbonate in 0.1N NaOH.
Solution B
0.5%copper sulphate in 1% sodium potassium tartarate.
Solution C
Mix 50 ml of A and 1ml of B to give alkaline copper solution.
FC Reagent
Protein standard
1mg/ml of standard BSA.

Procedure

- Aliquots of protein solution were pipetted out ranging from 0.2 to 1ml into clean test tubes.
- The volume was made upto 1 ml with distilled water.
- 5 ml of alkaline copper was added to each of the test tubes.
- It was mixed well and allowed to stand at room temperature for 15 min.
- 0.5 ml of FCR was pipetted into each tube and incubated at room temperature for 30min.
- The blue color formed is read at 650nm.
- A graph of concentration of protein Vs OD was plotted and the unknown conc. of protein determined.

Result: the amount of protein in the given sample was found to be…..

Observations

The amount protein present in the given sample is µg/ml

Viva-Voce

1. How do you estimate the protein content in the given sample?
2. Explain the principle of Lowry's method?
3. What is the protein that should be used as standard protein in protein estimation?
4. Which are the chemicals used in the estimation of protein content?
5. What is BSA and its importance?

Experiment No: 09 Date:

DEVELOPMENT OF INNOCULA: LAG TIME EFFECT

Aim To determine the effect of inocula concentration on lag time of bacterial growth.

Introduction; Bacteria are small, around 0.5 to 1.0 micron in diameter. The shape of the bacterim is governed by its rigid cell wall. The bacilli are straight rods that occur singly or in pairs. *Bacillus subtilis* form chains, When bacteria are inoculated in a suitable liquid medium and incubated the cell population will go through a number of phases represented as growth curve consisting of lag, log stationary and death phase.

Requirements
Well grown cultures, conical flasks, gyratory shaker, incubator, deep freezer.

Procedure

- The cultures maintained on an agar slant is used as a stock culture for the experiment.
- 2 ml of suspension is aseptically inoculated into 5 different flasks each containing 30ml of synthetic media.
- The cultures are maintained at 120 rpm at 32^0 C.
- Different innocula concentration of bacteria is achieved by arresting the growth of bacteria in culture flasks by removing one flask at a time from the shaker at 1 h interval and transferring to a deep freezer at – 20^0 C.
- After all the concentrations of inocula is obtained, 5 ml of inocula from each stock of 1, 2, 3,4,and 5 h grown cultures are transferred to 10 culture flasks containing 30 ml of media.
- The OD is read in all flasks at 1 h interval and growth curve plotted for all the 5 concentrations of inocula.
- Growth curve graphs are compared to the 5 inocula concentrations to determine the inocula concentration with least lag time.
- The enumeration of bacteria in the above cultures is done by using pour plate method.

Observations

Result: Size of the organism at 0th time of inoculation =

Size of the organism after 30 minutes =

Development of Inocula: Lag time effect

1st Hour Inhibition	
Time (Hrs)	OD at 540 nm
1	
2	
3	
4	
5	

2nd Hour Inhibition	
Time (Hrs)	OD at 540 nm
1	
2	
3	
4	
5	

3rd Hour Inhibition	
Time (Hrs)	OD at 540 nm
1	
2	
3	
4	
5	

4th Hour Inhibition	
Time (Hrs)	OD at 540 nm
1	
2	
3	
4	
5	

5th Hour Inhibition	
Time (Hrs)	OD at 540 nm
1	
2	
3	
4	
5	

Experiment No:10 Date:

SHAKE FLASK STUDIES: COMPARISON OF SYNTHETIC AND COMPLEX MEDIA

Aim: To determine the effect of gyratory shaking on cell mass formation and to compare the yield in synthetic and complex media

Requirements

Bacterial cultures, conical flask, gyratory shaker, incubator, simple media and complex media.

Complex media

Beef peptone broth
Peptone- 5gm
Beef extract-3gm
Dextrose-10gm
Yeast extract-5gm
Distilled water-1 l
pH- 7.4

Simple media

Peptone- 5gm
NaCl- 5gm
Distilled water- 1 l
pH- 7.4

Procedure:

- Working stocks of bacterial cultures were made in nutrient broth.
- 2 ml of actively growing cultures were inoculated into 10 flasks each containing synthetic medium and complex medium. Separate controls were maintained for simple and complex medium.
- One set of cultures inoculated into simple, complex and controls was transferred into gyratory shaker maintained at 180 rpm and incubated at 37^0 C.
- One set of flasks were incubated in an incubator at 37^0 C without shaking to determine the effect of non shaking on cell mass formation.
- Optical density was read in all flasks at the end of every 1 h of incubation.
- A growth curve of OD Vs time was plotted for bacterial growth in the shaking and non shaking cultures.
- All graphs were compared to determine the effect of cell mass formation in simple and complex medium and the effect shaking and non shaking.

Observation:

Complex Media		
OD at 540 nm		
Time Hrs	**With shaking**	**Without shaking**
1		
2		
3		
4		
5		

Simple Media		
OD at 540 nm		
Time Hrs	**With shaking**	**Without shaking**
1		
2		
3		
4		
5		

Sl No	**Volume of Protein solution(ml)**	**Volume of water (ml)**	**Volume of Alkaline copper (ml)**		**Vol of FCR (ml)**		**Conc. Of protein µgm**	**OD at 650 nm**
1(B)	0.0	1.0	5ml	Incubate in room temp for 15 min	0.5 ml	Incubate in room temp for 30 min		
2	0.2	0.8						
3	0.4	0.6						
4	0.6	0.4						
5	0.8	0.2						
6	1.0	0.0						
7	1.0 (unknown)	0.0						

Viva-Voce

1. What is the use of shake flask studies?
2. What is the difference between synthetic and complex medium?
3. Give one example for synthetic and complex medium?
4. Explain the principle of shake flask studies?
5. Name any compound which will be obtain from similar culture?.

Experiment No: 11 Date:

PRODUCTION AND BY ESTIMATION OF CITRIC ACID FROM *Aspergillus niger*

Introduction: Citric acid is an important organic acid and it was initially being extracted from citrus fruits. Nowadays it is largely produced by microbial fermentation. Citric acid is commercially used in foods, soft drinks, pharmaceuticals, leather tanning, electroplating etc. *Aspergillus niger* is the most commonly used species for the production of citric acid. Most strains of *Aspergillus niger* which are mutants cannot oxidize citric acid and hence accumulate in culture medium. The composition of the culture medium is critical for obtaining high yield of citric acid. It is essential to limit the growth of the fungus, so that high yield of citric acid accumulates in the medium. this can be accomplished by keeping trace metal deficiency in the medium. Acid is added to achieve low pH of 3.5. Sucrose serves as a carbon source for the production of citric acid. Ammonium nitrate is used to prevent the fermentation of oxalic acid glutamic acid. Fermentation is aerobic and can be carried out by submerged culture method.

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Materials and Methods

Culturing *Aspergilus niger*

Prepare the citric acid medium & dispense about 50ml in 250ml conical flask. Autoclave and allow it cool. Inoculate the medium with spores of *Aspergillus niger* and incubate it on a shaker water bath at 25 °C with gentle shaking for 3-5 days. After Incubation, filter the mycelium using double layered muslin cloth & measure the amount of citric acid in the filtrate by colorimetric and titrimetric methods.

Citric Acid Production Medium

Sucrose - 150 g
Ammonium nitrate - 2.5 g
Potassium Dihydrogen Orthophosphate - 1.0 g
Magnesium sulphate heptahydrate - 0.25 g
Distilled water - 1L, pH - 3.5

Estimation Citric Acid by Titrimetric Method

The filtrate obtained is titrated against an alkali of known strength using phenolphthalein as indicator.The end point is the formation of pale pink colour. The volume of alkali used for neutralization is used to find the normality and the percentage of acid in the sample.

1. 100ml of the filtrate is pipetted into a conical flask and 2-3 drops of phenolphthalein indicator is add to it.
2. This is titrated against 0.1N NaOH taken in the burette till a pale pink colour is formed.
3. The titration is repeated till concordant values are obtained.

Calculation:
Example of Titration Results

No	Initial Burette Reading	Final Burette Reading	Vol of NaOH used
1	0	8.0	8.0
2	0	8.1	8.1
3	0	8.1	8.1

Normality of Citric acid = [N(NaOH) * V(NaOH)] / V(Citric acid)
= [0.1 * 8.1]/ 10
= 0.081

% of Citric acid = [Normality * Equivalent wt of citric acid * 100] / Volume of filtrate
= [0.081*96*100] / 10
= 77.76%

N - Normality, V - Volume.
Equivalent wt of Citric acid - 96.

Observations and Result

Viva-Voce

1. How do you produce citric acid?

2. What are the chemicals used in the estimation of citric acid?

3. Give the scientific name of organism that is used in the production of citric acid?

4. Explain the principle of production of citric acid?

5. What is the use of citric acid?