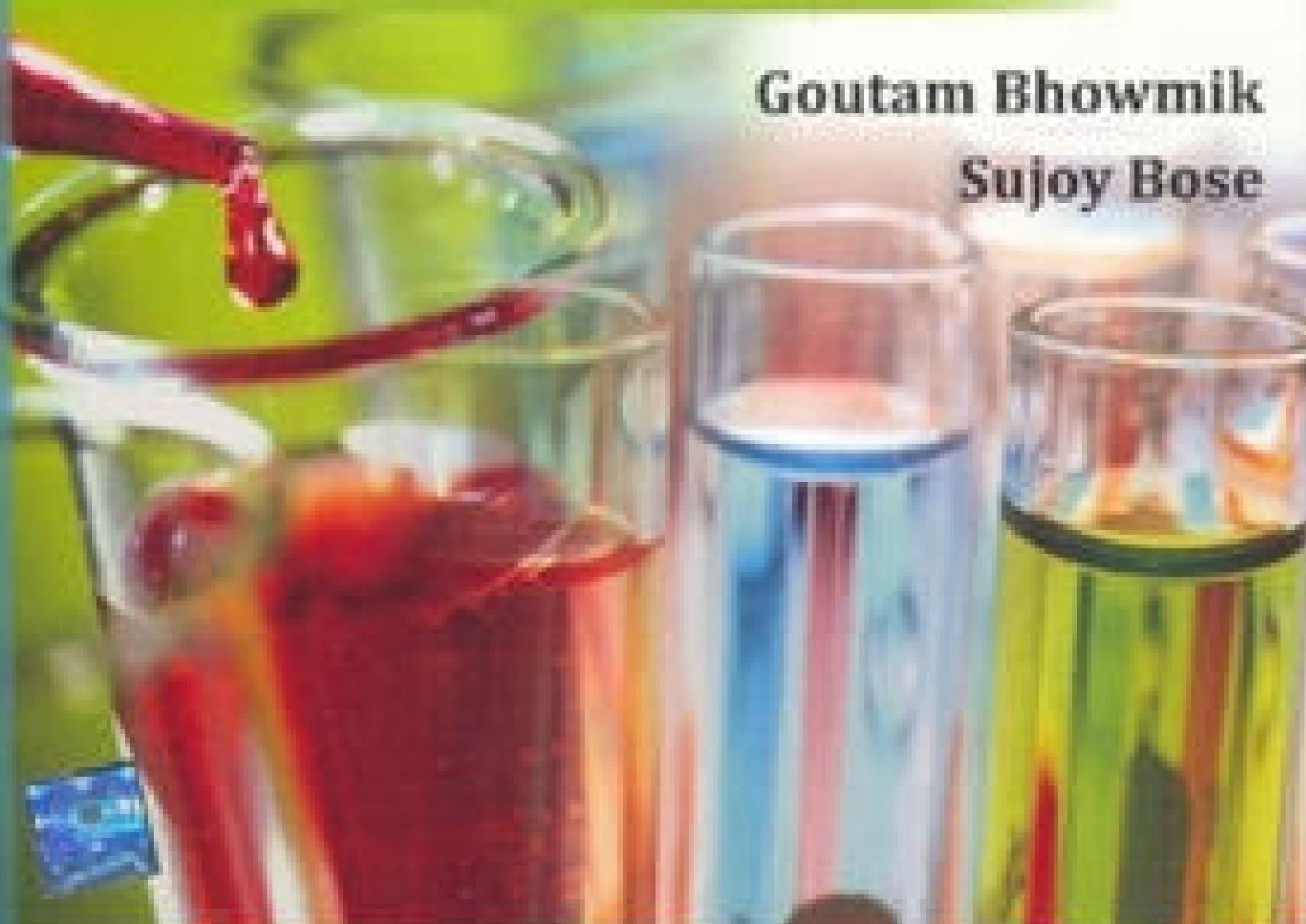


A COMPLETE LABORATORY MANUAL

Analytical Techniques in Biotechnology

Goutam Bhowmik
Sujoy Bose



Analytical Techniques in Biotechnology

(A COMPLETE LABORATORY MANUAL)

Author's Profile



Goutam Bhowmik received his BSc in Agriculture (1963) and MSc in Agriculture (1965) from Banaras Hindu University (BHU), Varanasi. He then obtained his PhD in Genetics and Plant Breeding from BHU, where his field of research was in micro-mutational and cytogenetical studies on barley.

His first work assignment was as a Lecturer at BHU from 1966 to 1969. From 1970 to 2004, he was a faculty member at Gauhati University, Guwahati (Assam). He has held the position of lecturer, reader, professor and HOD in the Department of Biotechnology, and was also a member of the Academic and Executive Council of Gauhati University. Fifteen students have received doctorate degrees under his supervision and guidance. His research papers are focused on tissue culture and isolation of mutations in pineapple, orchids, tea, pigeon pea and French beans. Moreover, he has standardised *in-vitro* propagation of many medicinal plants.

An eminent academician, Dr Bhowmik has published 45 and 32 research papers in national and international journals, respectively. He has visited many European countries as a participant of the Cultural Exchange Programme, Government of India and has conducted many training programmes as well as research projects sponsored by DBT, ICAR, CSIR, G B Pant Institute and ASTEC (Assam). He was a member and office bearer of various national scientific societies. He was the organiser of a DBT sponsored national seminar on 'Patenting in Biotechnology' and has also conducted several other seminars in the field of Biotechnology.

His authorial activities include being the adaptation author of a book on Genetics (4th Ed.) and editing a book on Patenting.



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Analytical Techniques in Biotechnology

(A COMPLETE LABORATORY MANUAL)

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Dedicated to

*Abira, Ruchira and
Ananya*

Contents

Preface
Abbreviations

xi
xiii

I: Introduction to the Laboratory

I

- Basic Pre-Laboratory Preparations 2
- Biology Lab Safety Rules 2
- Philosophical and Ethical Issues 5
- Suggestions to the Instructor 6
- General Information for Keeping a Laboratory Notebook 7
- Laboratory Reports 7
 - (a) Aim or Objective 7
 - (b) Basic Principle in Brief 7
 - (c) Materials or Requirements 7
 - (d) Methods or Procedure 7
 - (e) Data Tabulation or Observation 7
 - (f) Result(s) or Finding(s) 7
 - (g) Figure(s) and Finding(s) 7
 - (h) Discussion and Conclusion(s) 7
 - (i) Special Precautions (if any) 8
 - (j) References 8
- Ideal Laboratory Conditions 8
- Operation Techniques of Some Equipment and their Basic Working Principles 9
 - (a) Pipetting with Pipetman 9
 - (b) Measuring Absorbance with a Spectrophotometer 10
 - (c) Preparing Dilutions 11
 - (d) Buffer and pH Determination 12
 - (e) Metric Length and Fluid Volume 12
 - (f) Using a Microscope 13
 - (g) Binomial Nomenclature 15
 - (h) Centrifugation Technique 15
 - (i) Polymerase Chain Reaction (PCR) 17

viii Contents

- (j) Electrophoresis Technique 20
- (k) Sterilization Techniques 25
- (l) Autoclaving 27
- (m) Distillation 28

2: Genetics

31

- Expt. 1 Study of various stages of Meiosis and Mitosis calculating Mitotic Index 31
- Expt. 2 Preparing Karyotype of human chromosomes from photograph 36
- Expt. 3 Problems in Mendel's Law of Dominance, Segregation and Independent Assortment 38
- Expt. 4 C-banding technique of *Zea mays* pachytene chromosomes 43
- Expt. 5 Problems in Incomplete Dominance Modified Ratios and Multiple Alleles 45
- Expt. 6 Problems in Linkage 52
- Expt. 7 Problems in Sex-determination 58
- Expt. 8 Problems in Quantitative Inheritance 63
- Expt. 9 Estimation of Genetic parameters and partitioning of variance 66
- Expt. 10 Problems in Probability, Test of Significance (Chi-Test) and Hardy Weinberg's Law 71

3: Microbiology

78

- Expt. 1 A. Isolation of bacteria from Curd, Root nodules, Soil and Water samples 78
B. Isolation of bacterial and fungal spores from 'Air samples' 81
- Expt. 2 Aseptic technique and transfer of microorganisms for pure culture 83
- Expt. 3 A. Staining Gram Positive (+ve) and Gram Negative (–ve) bacteria 85
B. Staining bacterial endospore 88
- Expt. 4 Determination of bacterial growth curves by
(a) Manual counting (by microscope), and
(b) UV-Spectrophotometer method 89
- Expt. 5 Identification of bacteria through Biochemical test and Pigmentations 91
- Expt. 6 Uses of Physical and antimicrobial agents to control microorganisms 95
- Expt. 7 Determine which type of garbage is Bio-degradable, and which are non-degradable 102
- Expt. 8 Isolation and Identification of Non-Pathogenic Fungi 105
- Expt. 9 Determine the cell viability by dye-exclusion method 107
- Expt. 10 Plaque count by infecting the bacterium *Escherichia coli* B with its specific bacteriophage Coliophage T4. (See Chapter VII; Expt. 8) 109

4: Plant Tissue Culture

114

- Expt. 1 Preparation of Stock solution for MS basal medium and Plant Growth Regulator Stock 114
- Expt. 2 Aseptic culture technique for establishment and maintenance of carrot root cubes and Tobacco plant by leaf disc culture 118
- Expt. 3 Aseptic organ culture technique, of embryo and ovary 121
- Expt. 4 Determine callus growth and calculate the Callusing Index 123
- Expt. 5 Study the effect of auxin or cytokinin and the plant growth regulators 126
- Expt. 6 Micro-propagation of rice by *in vitro* organogenesis from embryo 129
- Expt. 7 Indirect somatic embryogenesis in carrot (*Daucus carrota*) 131
- Expt. 8 A. *In vitro* germination of orchid seeds 133
B. *In vitro* germination of Tobacco, and Tomato seeds 135
- Expt. 9 Exact protoplast from plant tissue and demonstrate the somatic cell fusion 137
- Expt. 10 Production of synthetic seeds and test their viability 140

5: Biochemistry, Enzyme and Fermentation Technology 145

- Expt 1A Estimation of mouse or chicken liver glycogen by anthrone method 145
- Expt 1B Preparation of liver homogenate and estimation of liver glycogen 147
- Expt 2 Extraction of lipid from egg yolk 148
- Expt 3 Separate lipids by thin layer chromatography (TLC) method 149
- Expt 4 Protein extraction and estimation from mouse liver 150
- Expt 5 Preparation of standard curve of protein (BSA) by Folin-Lowry method and determination of unknown protein concentration 151
- Expt 6 To study the effect of enzyme lactase on milk 153
- Expt 7 To immobilize yeast cells by sodium alginate entrapment method and to study the rate of conversion of glucose by immobilized yeast cells 155
- Expt 8 Protease estimation from fruits 157
- Expt 9 Extraction of ethyl alcohol from molasses by yeast fermentation 159
- Expt 10 Sauerkraut production and to analyze certain parameters during production (the period of fermentation) 161

6: Cell and Molecular Biology 165

- Expt 1 Estimation of nucleic acid. To estimate the RNA isolated from a given sample
OR Spectrophotometric analysis of RNA/ orcinol determination of RNA 165
- Expt 2 Estimation of DNA by diphenylamine (DPA) method.
OR Preparation of DNA standard curve through spectrophotometer analysis using diphenylamine reagent 167
- Expt 3 Extraction of Genomic DNA. To extract genomic DNA from a given plant specimen by using CTAB method (Based on method of Saghai- Maroof *et al.*, 1984) 168
- Expt 4 DNA extraction from blood 170
- Expt 5 Perform agarose gel electrophoresis for screening the DNA isolated and to estimate the relative sizes 172
- Expt 6 RNA isolation and cDNA preparation.
A. To isolate total RNA from liver tissue by Trizol method 174
B. Complimentary DNA (cDNA) preparation from the isolated total RNA 175
- Expt 7 Fractionation of cellular components-
A. Isolation of mitochondria. 176
B. Isolation of chloroplasts from a given plant specimen 177
- Expt 8 Separation of proteins by SDS gel electrophoresis 178
- Expt 9 Blood smear preparation and screening. To prepare a blood smear and to study the types of cell present 181
- Expt 10 Problems in molecular genetics 183

7: Recombinant DNA Technology 186

- Expt 1 Bacterial Transformation. Competent cell preparation. Bacterial transformation and screening of transformed colonies 186
- Expt 2 Isolation of plasmid. Plasmid alkaline lysis-miniprep protocol 188
- Expt 3 Detection of plasmid DNA by agarose gel electrophoresis 189
- Expt 4 Restriction digestion of DNA and its analysis by agarose gel electrophoresis 191
- Expt 5 Polymerase chain reaction—To set up a standard PCR reaction for a specific gene fragment of interest 193
- Expt 6 Gene cloning—To clone a gene of insert in a suitable vector system 194

x Contents

- Expt 7 Bacteriophage isolation—To isolate bacteriophage/coliphages from the sewage sample 196
- Expt 8 Transformation by co-cultivation techniques in tobacco plants (leaf discs) using *Agrobacterium tumefaciens* 197
- Expt 9: Medical Biotechnology—To separate blood mononuclear cells by Ficoll hypaque and observing viable PBMC's by trypan blue staining 201
- Expt 10 Bioinformatics
- A. To analyze a given gene sequence for mutation by software analysis 202
 - B. To analyze a given protein sequence for mutation by software analysis 205

8: Animal Biotechnology and Immunology 207

- Expt.1 To learn the basics of animal cell culture 207
- Expt. 2 To describe the maintenance of cells in culture 208
- Expt. 3 To estimate the number of viable cells using the trypan blue viability test method 210
- Expt. 4 Protein extraction and estimation from cell lines by immuno-blotting 211
- Expt. 5 Immunology. Bacterial antigen preparation—Preparation of antigens from bacterial culture routes of inoculation of antigens in rabbit/mice by different routes of immunization 213
- Expt 6 Screening for the development or formation of the primary antibody against the inoculated (administered) antibody by-
- A. 'Haemagglutination' 215
 - B. 'Immuno-diffusion' methods 215

Annexures 218

- Annexure I List of Equipments required for conducting Biotechnology practical 218
- Annexure II Common Buffers usually used in Biotechnological experiments 218
- Annexure III Stains and Nutrient Media composition for Microbiological experiments 220
- Annexure IV Plant Tissue Culture Media Composition 226
- Annexure V Answers to the Problems in Chapter II: Genetics and Chapter VI: Cell and Molecular Biology 228
- Annexure VI Chi-square table (χ^2) 234
- Annexure VII References/ Technical Literatures 234
- Annexure VIII Addresses of Some Suppliers of Equipments (Minor and Major), Chemicals, Glass- and Plastic-wares 235

Index 237

Preface

The identification and study of human genes pose one of the greatest challenges to the past and the future of mankind. In India, two streams of work with genes seems to have come to the forefront, putting Indian geneticists on the global map. First, a consortium of Asian Scientists, coordinated by the Genome Institute of Singapore, has analysed variations in genetic sequences from which it may be concluded that all Eastern Asian population had roots in India. Secondly, the scientists at the Institute of Genomic and Industrial Research claim to have successfully mapped the human genome sequence for the first time in India, thereby joining, albeit a bit late in the day, an exclusive club of five nations. The benefits of this achievement are more directly related to the field of medicine, from predicting diseases in individuals to advanced forms of treatment.

Need for Quality Manpower

This necessitates grooming of personnel from grass-root levels for quality biotechnological research in order to cope up with the fast-changing scenario. We believe that emphasis on efficient practical applications leads to a better understanding of theoretical knowledge.

Leaving aside some private research laboratories, a few elite central universities, IITs and NITs, other institutes across the country need significant up-gradation. Recently, 374 world-class universities have been permitted to set up campuses countrywide (UGC newsletters) which will create a vacuum for quality biotechnologists [students, professionals, researchers and scientists], until this gap is bridged by trained hands.

Target Audience: Conceiving the Idea

Keeping in mind the present scenario of practical-class standard in colleges and universities, we conceived the idea of taking up this project with Tata McGraw Hill Education. This book will be useful for any fundamental course of Biotechnology, e.g., BSc Biotechnology, BTech Biotechnology and MSc Biotechnology. It will cater to all major papers of other undergraduate and postgraduate life-science courses like BSc Botany, BSc Zoology, BSc Biochemistry as well, where Biotechnology is an ancillary subject. Besides, it can be helpful to XI and XII CBSE students opting for Biotechnology as a subject.

Organisation of the Book: Selection of Practicals

It was done with the hope of emphasising standard work and including cost-effective experiments, so that students may perform a large number of useful biotechnological practicals. We are aware that there is ample scope for including innumerable number of experiments, but only those experiments which are of significant importance have found place in this book. Biotechnological subjects like Genetics, Microbiology, Plant and Animal Tissue Culture, Biochemistry, Enzyme and Fermentation Technology, Immunology, Cell and Molecular Biology, Recombinant DNA Techniques, Food, Environment and Medical Biotechnology have been included.

The book consists of eight chapters covering experiments on the above topics, to give an essence in application of biotechnology compiled in a single book. More than 20 syllabi of different universities, IITs and other renowned institutions have been scrutinised in selecting these experiments.

xii Preface

The experiments are concisely structured to cover all requisite details. The language is simple and lucid. For easy understanding, the Table of Contents is segregated into 6 broad sections: Common Laboratory Techniques, Genetics, Microbiology, Plant Tissue Culture, Cell and Molecular Biology, Recombinant DNA Technology and Biochemistry.

Besides this, each experiment is explained stepwise with features like

Key terminologies, Aim, Principle/Theoretical Background, Materials required, Examples, Solved Problems, Step-wise Protocol, Notes, Figures, Data Tabulation—how to record observations, how to draw conclusions, Precautions, and Frequently Asked viva-voce Questions. Techniques for low-cost methodologies to perform experiments are also given.

The strong pedagogy includes

- ❑ Over 50 relevant photographs and illustrations
- ❑ More than 100 solved and unsolved problems
- ❑ Over 200 viva-voce questions
- ❑ 8 appendices

Scope and Choice of Experiments

This book will give a wide scope in selecting the practical experiments by teachers and instructors according to their budget and institutional facilities. It will serve as a readymade reckoner to the teachers, and students can learn how to conduct and develop a practical step by step. Each of these experiments also includes **key terminology** and **basic principles** underlying them besides possible viva questions.

Acknowledgements

I (Goutam Bhowmik) am indebted to my wife, Dr Sudipa Bhowmik, and daughters Abira and Ruchira for their constant encouragement and inspiration. Without their support, it would not have been possible to complete this book. I am also grateful to my student Dr Sujoy Bose, for framing, writing and suggesting constructive ideas throughout. We would also like to thank Mr Kunal Hore for helping with the illustrations. Also, some reviewers of the book deserve a special mention for their useful suggestions and comments. Their names are given below.

Partha Roy	<i>Indian Institute of Technology (IIT), Roorkee</i>
J Kamalesvari	<i>Pachiyapas College of Arts and Science, Chennai</i>
J Joel Gnanadoss	<i>Loyola College</i>
Pankaj K Hiradhar	<i>Rajasthan University</i>

Feedback

We are also aware of the fact that there may be many shortcomings in the book. Therefore, we without any prejudice, invite constructive criticisms and useful suggestions from the users. This will also help us to improve the future editions.

GOUTAM BHOWMIK

SUJOY BOSE

Publisher's Note

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Piracy-related issues may also be reported.

Abbreviations

APS	Ammonium per sulphate	MI	mitotic index
aq	aqueous	min	minutes
B ₅	Gamborg et al.	ml	millilitre
Bis	N, N-methylenebisacrylamide	mm	millimetre
BAP	benzyle amino purin	mM	millimolar
bp	base pair	MS	Murashighe & Skoog
BSA	bovine serum albumin	MW	molecular weight
cm	centimeter	MSDS	material safety data sheet
cM	centimorgan	ng	nano gram
CTAB	cetyltrimethylammonium bromide	nm	nanometer
2,4-D	2,4 dichlorophenoxy acetic acid	NAA	1-naphthyl acetic acid
DDH ₂ O	double distilled water	O.D.	optical density
DNA	deoxyribonucleic acid	PAGE	polyacrylamide gel electrophoresis
EDTA	ethylenediamine triacetic acid	PCR	polymerase chain reaction
emf	electromotive force	PMC	pollen mother cell
EtBr	ethidium bromide	PMSF	poly methyl sulfonate
ε	molar extinction coefficient	pNP	p-nitro phenol
FDA	flourescein diacetate	pNPP	p-nitro phenyl phosphate
g	gram	RBC	red blood cells
g/l	gram per litre	RE	restriction enzyme
GA ₃	gibberellic acid	RNA	ribonucleic acid
h	hour	rpm	revolution per minute
IAA	indole-3- acetic acid	RT	room temperature
IBA	indole-3- butyric acid	SDS	sodium dodecyl sulfate
kbp	kilobase pairs	sec	second
KIN	kinetine	SSC	saline solution citrate
l	litre	TE	tris-EDTA
LB	Luria Bertini	TAE	tris-acetate-EDTA
lb/in ²	pounds per square inch	TBE	tris-borate-EDTA
mA	milliampere	TEMED	tetramethylethylenediamine
μ	micron	Tris-Cl	tris (hydroxymethyl) aminomethane Chloride
μg/μl	microgram per microlitre	UV	ultra violet light
μl	microlitre	V/V	volume per volume
μM	micromolar	W/V	weight per volume
μmol	micromoles	WBC	white blood cells
M	molar	xg	gravity
mg/l	milligram per litre		
mg/ml	milligram per millilitre		

1

Introduction to the Laboratory



Key Terminology

- 1 **Biotechnology** Biology is the study of living things, which are made of cells. Technology is about solving problems and making things. Biotechnology uses organisms, cells or parts, to make and do things we need. These cells may come from microbes, plants or animals.
- 2 **Analytical techniques** To know about solving problems critically or make logical reasoning.
- 3 **Application of practical knowledge/experience** Use of knowhow or experience of practical for useful purpose and utility.
- 4 **Laboratory direction** Procedures to be maintained or followed in the practical classes.
- 5 **Safety rules** Steps to be followed for safeguarding oneself and others.
- 6 **Biohazards** Harmful effect which may be caused by microbes or anything which comes from a living things, viz., animals or plants.
- 7 **Experimental evidence** Gathering information indicating whether a proposition is true or valid after working out a practical or problem or investigation.
- 8 **Interpreting results** Find out logical reasons or knowhow for solving any outcome of a problem.
- 9 **Inquisitiveness** Eagerly seeking knowledge or show interest to know something.
- 10 **Laboratory notebook** Practical copybook used for recording the findings or results obtained from doing practical work.
- 11 **Instrumentation** The instruments used for measuring some information.

The purpose of this laboratory manual is to help you learn fundamental laboratory techniques and good laboratory practices that are important in any bioscience laboratory. This course is intended to introduce you to some of the most widely used experimental procedures in Biotechnology. The development of Biotechnology is dependent on analytical techniques, and a wide variety of tools and sophisticated equipments. The emergence of the science of Biotechnology is due to the combined efforts of chemists, physicists, biologists, statisticians and engineers. Biotechnology is a multidisciplinary field having its roots in the biological, chemical and engineering sciences

leading to a host of specialties, such as molecular genetics, microbial physiology, biochemistry, biochemical engineering, etc., (Fig.1.1). It probes into the living cells or its constituents to make products or processes of commercial value. You will also gain familiarity with some of the types of equipment frequently used in biotechnology. The biotechnology laboratory course, like all laboratory courses, is an exploration of procedures.

Implementation of this manual has been conceived with an idea that biotechnological concepts depend much on practical guidelines and experiments. Understanding of the theoretical concept in biotechnology can come through

2 Analytical Techniques in Biotechnology

protocols and practical knowledge. Quality work requires thoughtfulness, an ability to recognize problems and a willingness to solve them. These are skills and aptitude that are invaluable in any scientific career.

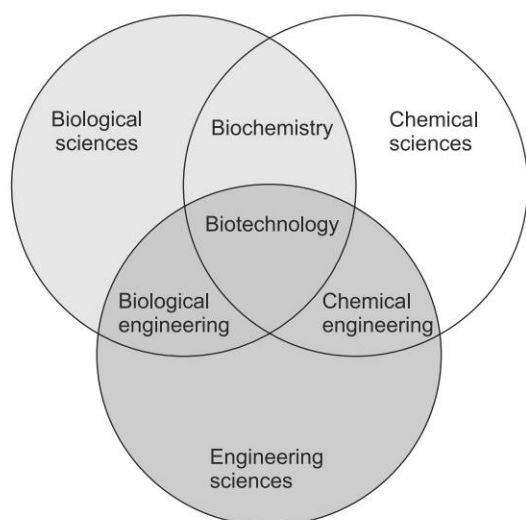


Fig. 1.1 Multidisciplinary nature of biotechnological science



BASIC PRE-LABORATORY PREPARATIONS

- Prior planning and conceiving a clear theoretical background is essential before performing any experiment.
- Go through the instructor's remarks carefully and be thorough with the practical procedures.
- Try to understand why a particular practical experiment has been included and how it is related to the subject.
- Analyze its applications and methods to use the results in Biotechnology.
- Be sure that all chemicals, glassware, tools and equipment are readily available.
- Make a list of chemicals, reagents, tools, glassware, and equipment needed before you begin an experiment and check whether these materials are readily available in the lab or not.

- Ensure better understanding of the material by acknowledging the problems related to the planned experiments.
- Read the manual and participate as much as possible in the discussions in order to get full benefit from the course.
- Ask questions in or out of the class. You should also try to participate in the actual lab work.
- The more effort you put into the course work, the more you will learn. A practical class is an opportunity to learn valuable skills.
- When in doubt, ALWAYS ASK FOR HELP.



BIOLOGY LAB SAFETY RULES

For the safety and convenience of everyone working in the laboratory, it is important that the biology lab safety rules are observed at all times.

The following rules need to be observed.

(a) Back to the Basics

- Place only those materials required for the day on the benchtop for performing the experiment. All other extra things should be placed in the lab bench storage areas under the lab benches in order to avoid damage or contamination.
- Eating, drinking, applying cosmetics or any other hand to mouth activity in the lab should be avoided.
- Unlabelled material found in the refrigerators, incubators or freezers should be destroyed. Always mark the back of the plates with your initials, the dates and relevant experimental data, e.g. strain number.
- Please read the laboratory exercises and follow the laboratory directions carefully. Familiarize yourself in advance with the exercises to be performed.
- The biology lab is an important aspect of any biology course. In order to have a good lab experience, make sure that you follow these biology lab safety rules and any instructions given to you by your lab instructor.

- Biology lab safety rules are guidelines designed to help keep you safe when experimenting. Some equipment and chemicals in a biology laboratory can cause serious harm.
- It is always wise to follow all lab safety rules. Don't forget, the most helpful safety rule is to use plain common sense.
- The following biology lab safety rules are a sample of the most basic rules that should be followed when in biology lab. Most biology labs have the safety rules posted in the lab and your instructor will most likely go over them with you before you begin working.
- Since you will use common facilities, all solutions and everything stored in an incubator, refrigerator, etc. must be labelled.
- Before you enter a biology lab, you should be prepared for and be knowledgeable about any lab exercises that are to be performed. That means you should read your lab manual to know exactly what you will be doing.
- Review your biology notes and relevant sections in your biology book before your lab begins. Make sure you understand all procedures and purposes, as this will help you understand the lab activities you will perform. It will also help you get your thoughts organized for when you have to write your lab report.
- Always read the label on a reagent bottle carefully to make sure it contains the chemical you want. Put the bottle in its original place immediately after use.

(b) Clothing

- Some chemicals have the potential to damage clothing. Make sure that the clothing you wear is something you could do without if it becomes damaged. As a precaution, wear an apron or a lab coat.
- Wear proper shoes that can protect your feet in case something breaks. Sandals or open-toed shoes are not recommended.

(c) Accident and Injuries

- Report any cut, burn or other injuries to your instructor immediately.

- Handle all glassware carefully. Notify your instructor of any broken glassware. Do not pick up broken glassware with bare hands. Use the dustpan and brush and dispose of it in the bin labelled **Broken Glass**.
- While working with hazardous chemicals, wear gloves, face mask and use safety glasses or goggles during working with a trans-illuminator.
- Work in a way that protects as well as does not disturb your fellow workers and the environment outside the laboratory.
- In case of emergency, immediately contact your instructor and report how it happened.
- Report all accident and breakages to your instructor. If any chemicals come in contact with your skin or clothing, first wash the affected area with a large amount of water and then report it to your teacher.

(d) Handling Chemicals

- The best way for handling chemicals is to assume that any chemical you handle is dangerous. Be sure you understand what type of chemicals you are using and how they should be handled.
- Wear protective eyegear when handling chemicals.
- Safety goggles should always be worn when you are working with chemicals or any type of heating apparatus.
- Always handle a flammable liquid with great care and keep it away from naked flame.
- Always handle concentrated acids and alkalis with great care.

(e) Handling Glassware and Equipments

- Be sure you know where to find all safety equipment in the biology lab. These includes items such as the fire extinguisher, first-aid kit, broken glass receptacles, and chemical waste bins.
- Also be sure to know where the emergency exits are located and which exit route to take in case of an emergency.

(f) Handling Heating Substances

- Use the Bunsen burner carefully and with caution. Do not lean over it.
- Always adjust the Bunsen burner to give a luminous flame when not using it (or just simply turn it off).
- Remember to switch off the electric plug after you use an electrical heater.

(g) Cleanliness

- Since you have only a limited amount of space to call your own, it is to your advantage to keep your work area clean.
- After completing an experiment or when one lab class is over, dispose of all material properly and clean the table top with a disinfectant.
- All common areas should be kept free of culture and dirty glassware, electrophoresis equipment, etc. should be dealt with appropriately.
- Always clean the oil from the oil immersion lens of the microscope with a piece of lens paper or pure silk cloth and xylene.
- Return all equipment, reagents and other supplies to their respective places at the end of the class.
- Always wash and/or sanitize your hands with a disinfectant soap before leaving the lab.

(h) Handling Microorganisms or Cultures

- Some microorganisms (in microbiology experiments) used, may be pathogenic, therefore, it is essential to always follow proper aseptic techniques while handling organisms.
- If you spill a culture, observe the following procedure:
 1. Immediately throw the culture tube in the plastic bin labelled '**Bio-hazard**', so that no one else touches the contaminated tube.
 2. After putting off the Bunsen burner, spray isopropyl alcohol. After a few minutes, use paper towels to dry the area.
 3. Wash your hands with a disinfectant soap and sanitize your hand.

4. Notify your instructor about the spill.

- Place all inoculated material in an assigned place in the lab. Culture tubes should be stored upright in a stand or a plastic beaker, while petri dishes may be stacked and incubated upside down.
- To avoid contamination and distract attention from your experiment, do not use cell phones or other personal media devices in the laboratory.
- If body fluids or bloods are used in a laboratory experiment (e.g. blood grouping experiment), each student should work only with his or her own sample. All caution should be maintained to disinfect the punching needle. Remember to dispose of the used swabs, needles, etc. at the end of the experiment in a '**Bio-waste**' container.

(i) Biology Labs Do's and Don'ts

- Don't run or play in the laboratory
- Don't eat or drink in the lab
- Don't taste any chemicals or substances you are working with
- Don't use your mouth for pipetting substances
- Don't handle broken glass with bare hands
- Don't pour chemicals down the drain without permission
- Don't operate lab equipment without permission
- Don't perform your own experiments unless granted permission
- Don't leave any heated materials unattended
- Don't place flammable substances near heat
- Don't engage in childish antics such as horseplay or pranks
- Don't throw regular trash in the '**Biohazard**' container.
- Never enter the laboratory unless a teacher is present.
- Never remove anything from the laboratory without your teacher's permission
- Never use your bare hands to transfer chemicals. Use a spatula instead.

- Never leave experiments unattended.
- Never look directly down the test-tube or point the mouth of a test-tube towards anyone when heating.
- Never taste anything without your teacher's permission.
- Never put solids in the sink.
- Never use a laboratory microwave or freezer for food. Do not use laboratory glassware for eating or drinking.

(j) Special Safety Measures for Hazardous Substances

1. Chemicals A number of chemicals used in a molecular biology laboratory are hazardous. Follow these instructions carefully while handling the chemicals.

- Carefully read manufacturer's manual on any hazards associated with the chemicals supplied by them.
- Collect **Material Safety Data Sheets (or MSDS)** attentively where this information is provided.
- Take stock of the following information in the MSDS
 1. Chemical name of the substance
 2. Health hazard data
 3. First aid treatment
 4. Physical data
 5. Fire and explosion hazard data
 6. Reactivity data
 7. Spill or leak procedures
 8. Any special procedures while handling a particular chemical
- Paste the MSDS sheets on the wall near the entrance for quick observation.
- Assess the internet for any updates on the MSDS.
- Religiously use this information prior to using a new chemical and certainly in the case of any accidental exposure or spill.

The following chemicals are particularly noteworthy:

- Phenol—Can cause severe burns
- Acrylamide—it is a potential neurotoxin
- Ethidium bromide—It is carcinogenic.

These chemicals are not harmful if used properly: always wear gloves **when using potentially hazardous chemicals and never mouth-pipette them**. If you accidentally splash/spill any of these chemicals on your skin, **immediately rinse the area thoroughly with water**.

2. Electricity The voltages used for electrophoresis is sufficient to cause electrocution. Cover the buffer reservoirs during electrophoresis. **Always turn off the power supply and unplug the leads before removing a gel.**

3. Ultraviolet Light Exposure to ultraviolet light can cause severe eye damage. Since the retina cannot detect UV light, you can have serious eye damage and not realize it until 30 min to 24 hours after the exposure. **Always wear appropriate eye protection when using UV lamps.**



PHILOSOPHICAL AND ETHICAL ISSUES

Scientific research involves an exploration of the unknown. In some classes, a question has a 'correct' answer. However, in research, the correct answer is rarely known in advance, and must instead be inferred from the experimental results. **Researchers must always remain open to experimental evidence that contradicts a hypothesis.** Your task as a student of science will be to consider your data and interpret it. In this context negative results or answers are also answers, that may be contradictory to your data or do not arise logically from the data you have collected.

This uncertainty as to the 'correct' answer means **that you must be careful when reporting what you did, how you did it and what you observed**, especially if you observe something unexpected. To avoid embarrassment after publishing a reaction or result is one of the reasons for the care that people take in performing experiments and interpreting the results. A scientist may publish a statement that, information in a previously published paper is the result of an artifact. Repetition of some experiments is, therefore, essential.

6 Analytical Techniques in Biotechnology

The sources of the information you have used must be properly cited. You should always properly recognize the authors, publishers or books you have consulted. It also means that you should cite the inventors of the methods that you have used for your experiments. You should approach the course with an open and flexible mindset.



SUGGESTIONS TO THE INSTRUCTOR

Much depends on the instructor to create inquisitiveness for a subject. Before commencement of

will work in pairs or in groups and what safety measures are to be followed, etc.

- The instructor should familiarize the students with warning labels for specific chemical hazards labels (Fig. 1.2).
- In the laboratory, proper bins for biological hazardous materials, broken glasses, microbial cultures or other organisms must be used and should be placed in or outside the laboratory at fixed locations for easy disposal (Fig. 1.3).
- The instructor should insist on clear and proper labelling on cultures, reagent bottles, etc.

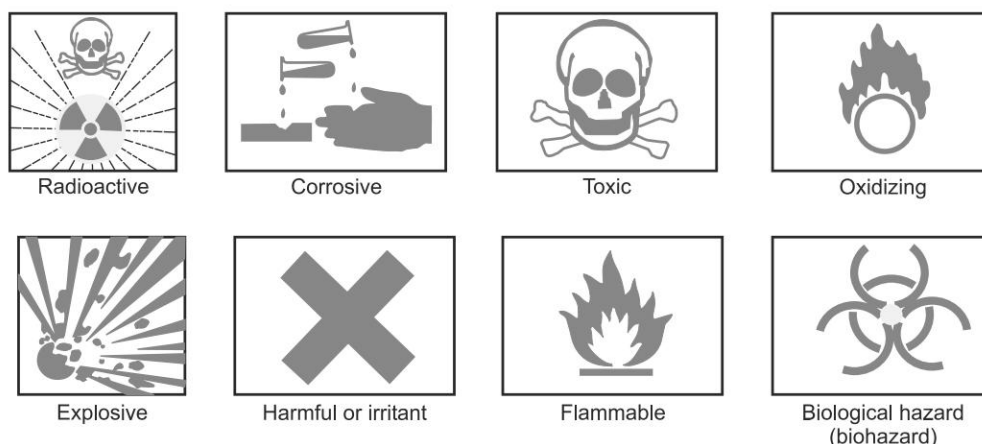


Fig. 1.2 Warning labels for hazardous chemicals

any experiment clear and specific instructions should be given to the students. Following are some of the points that may prove useful:

- It depends on the instructor to design or perform or select an experiment according to the facilities available in the college.
- It may be necessary to improvise or modify new experiments based on the results of previous ones, or to repeat experiments that yielded non-interruptible or ambiguous results.
- Before commencement of any experiment the instructor should clearly indicate what will be the protocol to be followed in conducting it, how much chemicals, glassware, tools, etc. are to be issued, whether the students
- He/She should insist on use of laboratory coats or aprons, hand gloves and safety-glasses wherever it is essential.
- The instructor should check the record notebook of each student either in pair or groups and countersign the record (after checking the observations).
- For safety and proper handling of costly tools and equipment, such as pipettes etc., the instructor should demonstrate the steps before the commencement of the experiment.
- Any or a combination of the following disciplinary action has to be taken by the instructor on an erring student engaged in such actions as to cause damage to college property; unsafe condition; injury to another person or disruption that interferes with learning:

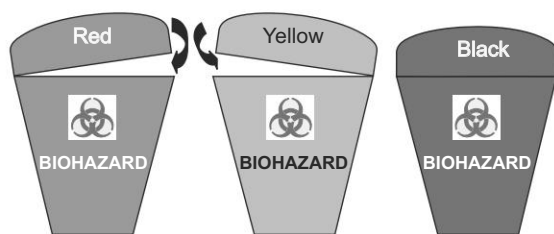


Fig.1.3 Disposal of different types of wastes from a biotechnological Laboratory. **Red Bin**—for highly contagious or genetically modified organisms, blood, disposable syringes, needles, etc. Has to be destroyed by an incinerator. **Yellow Bin**—Microbial wastes, laboratory wastes, to be autoclaved before disposing. **Black Bin**—Normal garbage. Can be disposed off normally

- (a) A verbal or written warning
- (b) Direct to leave the class for the remainder of the period or for the week
- (c) A referral to the principal or the departmental head.
- An undertaking must be obtained regarding safety rules stating—
 “I have read and understood the safety rules and policies of the college laboratory; I agree to follow the safety policies. I understand that failure to comply with the safety and laboratory guidelines may result in a reduction of my final grade and/or I may be asked to leave the class or college.”



GENERAL INFORMATION FOR KEEPING A LABORATORY NOTEBOOK

Maintaining a laboratory notebook is an art. The notebook will be useful for the recording of laboratory data calculation and help you for writing the lab reports. Instruction regarding keeping or maintaining the laboratory notebooks is, therefore, a major part of most laboratory courses. Each experiment should begin with a record of exactly what you did at each step (be sure to mention or make a note of anything that you do which differs from the information in the manual). You should also record any numerical information, such as the exact amount of reagents used, absorbance reading, enzyme activities, protein concentrations and buffer concentrations, etc. Most experiments

may extend over several days and over several pages of your notebook. To keep track of what you have done, it should include the date at the top of each page, including sub-titles for each page you used for each step. If you are writing something while in the laboratory, it should be written directly in the notebooks, including any calculations performed.



LABORATORY REPORTS

While writing laboratory practical reports, they should be written as follows:

(a) Aim or Objective Precisely state the purpose and objective(s) of the experiment in one or two sentences.

(b) Basic Principle in Brief Write the basic principle after reading the report. It is given before each experiment in this manual. If necessary, consult other textbooks.

(c) Materials or Requirements Clearly list out the equipment, glassware (with required numbers), tools, chemicals (mention quantity), reagents, plant material, culture or organism, etc. needed for the experiment.

(d) Methods or Procedure Briefly, stepwise, explain the procedure to be followed in the experiment to achieve the result. Record exactly what you did at each step.

(e) Data Tabulation or Observation Data must be clearly recorded in the notebook in a tabular form. The data (result) generated in the experiment must be shown to the instructor.

(f) Result(s) or Finding(s) Actual result(s) may be entered in a concise manner without any correction or overwriting.

(g) Figure(s) or Finding(s) Proper representation of results are necessary by figure(s), graph or drawing (as per requirement of the situation).

(h) Discussion and Conclusion(s) Under this heading, the results should be briefly interpreted or justified and conclusion(s) be drawn.

(i) Special Precaution(s) (if any) Some experiments may need special attention or precaution(s) for achieving correct results and safe completion of it, to avoid health hazards, viz. handling microorganisms, safe and economic use of costly chemicals.

(j) References All reading materials, lab manual and books that were consulted for the said experiment should be recorded along with name of the author(s), year of publication and publisher.



IDEAL LABORATORY CONDITIONS

(a) Initiative Taken by the Faculty

- Outstanding faculty teaching requirements.
- Faculty should consistently offer enough skills, equally competent theory and laboratory or field research guidance.
- The faculty should be able to assign specific and clear instructions, show equal skill in pinpointing the outcome of experiment results, and emphasize on writing the practical report.
- It is the responsibility of the faculty to enhance an assignment and carry forward the course curriculum within a scheduled time-frame.
- The faculty should have the proper knowhow of his/her available laboratory facilities and resources.
- The faculty should be able to provide references, help in library consultation and be equipped with basic information on the subject or equipment.
- The faculty should continuously monitor the progress and evaluate his/her success of the plan or his/her adopted methodology.
- Success of practical work will depend on the teacher's planning, creative thinking and interaction with students.

(b) An Excellent Updated Biological Curriculum

- An ideal biological curriculum should create inquisitiveness about the subject.

- The curriculum should be appropriate to provide additional ideas for developing a research aptitude.
- An ideal biology curriculum would be such that beside the regular course curriculum, the students learn about reproduction, genetics, cancer, ecology, environment, nutrition, etc.
- The organisms should be simple (that infer correct and clearcut results) to maintain, require a minimum of advanced instruments, and the experiments inexpensive.
- Facilities for interactive media and animation films are necessary for a biological laboratory.
- Proper maintenance of specimen, e.g. cultures, plant, microorganisms, animals, etc.
- Appropriate disposable system for biological wastes and bio-hazardous materials.

(c) Laboratory Environment

- Laboratories need to be flexible, open, expandable, adaptable, functional, efficient and most important **safe**. Ideally, the focus should be on functionality, health and safety, convenience, and energy conservation. There are new trends in lab designs today and these trends have greatly been influenced by modernization and innovation.
- An ideal lab environment is when resource facilities can be assessed transparently without any ambiguity.
- All users of the lab shall have a uniform environment.
- An ideal lab should have good electrical, gas, water, and first-aid arrangements and should maintain proper safety precautions.
- Disciplined, eager and knowledgeable students should know what is to be done and how to carry out the experiments as per instructions.
- Continuous progress of the laboratory facilities—It is necessary to build the facilities in the lab over a period of time gradually rather than overnight.
- Proper ventilation of air and gas. The lab should be fitted with exhaust-fans or air-

conditioners, and there should be appropriate places for equipments, racks for chemicals and storage facilities.



OPERATION TECHNIQUES OF SOME EQUIPMENT AND THEIR BASIC WORKING PRINCIPLES

(a) Pipetting with Pipetman

In most molecular genetics and biotechnological experiments, the ability to accurately and reproducibly measure and transfer small volumes of liquids is of utmost importance for obtaining accurate results. Generally, for volumes less than 1 ml the most common method of measuring liquid volumes involves the use of a device known as a Pipetman (it is a brand name) (Fig. 1.4). However, all such pipetting devices work on similar principles.

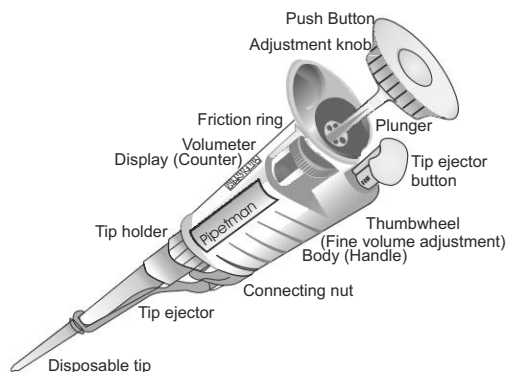


Fig.1.4 A typical Gilson's Pipetman

There are four basic types of pipetman—P1000, P200, P20 and 0.1–2.5 μl having different measuring ranges. P1000 is useful for volumes 100–1000 μl , P200 is useful for volumes 20 – 200 μl , P20 is useful for volumes 2–20 μl , while 0.1–10 μl is useful for volumes 0.1–2.5 μl . One may also opt for P10 pipetman which may be used for volumes between 0.5–10 μl . Be sure that you are using the correct Pipetman for the volume you need. Before measuring make sure that the Pipetman is actually set for the volume you are looking for in the “volume window”, and if necessary, turn the “volume control knob” until the pipetman displays the correct volume.

PIPETMAN No.	MEASURING RANGE
P1000	200 – 1000 μl
P200	50 – 200 μl
P100	20 – 100 μl
P20	0.5 – 20 μl
P10	1 – 10 μl
P2	0.2 – 2 μl

In all Pipetman, disposable plastic tip (do not pipette liquids without the appropriate tip) assemblies are used, because without it, the Pipetman may get contaminated and may spoil the experiment. Be sure that while attaching the tip, it is the right type for the Pipetman you are using and that the tip is properly seated on the end of the Pipetman.

As the plunger depresses, you will feel a sudden increase in resistance. This is the first stop. If you continue pressing the plunger, you will find a point where the plunger no longer moves downward (the second stop). While using the pipette, depress the plunger to the “first stop”, place the tip into the liquid and slowly, in a controlled manner release the plunger to move upwards (do not simply let the plunger go; doing so will cause the liquid to spatter within the tip, resulting in inaccurate volumes and contamination of the pipette).

Now take the pipetman (carrying the pipetted liquid in the tip) to the container or tube to which you wish to transfer. Press the plunger to the first and then to the second stop. If you watch carefully, you will notice that depressing to the second stop expels all the liquid from the tip. This is true for all aqueous solutions. However, in case of organic solvents or for solutions containing large amount of protein, it is difficult to get *either hold* or all the liquid out of the tip. In these cases, it is best to ‘wet’ the tip, by pipetting the original solution once, expel it, and then measure out the liquid again.

Precautions

Though Pipetmen are very useful, they have certain drawbacks—

- If used carelessly, Pipetman will transfer inaccurate volume.
- Very often Pipetman may lose calibration and yield misleading results.

- Regular checking of calibration of the Pipetman can save considerable time, energy and costly reagents.
- Do not attempt to set the Pipetman for volumes larger than their maximum, or for volumes less than 10% of the maximum permissible limit; doing so will damage the Pipetman.
- Always keep the Pipetman in the release mode, i.e. at maximum volume while not in use.

(b) Measuring Absorbance with a Spectrophotometer

A spectrophotometer is an instrument for measuring the absorbance of a solution. The Beer-Lambert law states that

$$A = \epsilon cl$$

where, A is the absorbance of the sample at a particular wavelength, ϵ is the extinction coefficient for the compound at that wavelength in $(\text{M}\cdot\text{cm})^{-1}$, c is the molar concentration of the absorbing species, and l is the path length of the solution in cm. As such, if the extinction coefficient of an absorbing sample is known, the absorbance of the solution can be used to calculate the concentration of the absorbing species in the solution (if it contains only one material). Generally light is used to measure absorbance.

Another definition of absorbance is

$$A = \log (I_0 / I)$$

where, I_0 is the amount of light entering the sample and I is the amount of light leaving the sample. Therefore, measurement of absorbance is $I -$ amount of light leaving the lamp (I_0) that actually reach the detector (I) after transmission. When absorbance = 1, only 10 per cent of light is reaching the detector; when it is = 2, only 1 % of the light is reaching the detector (Fig. 1.5).

Absorbance value greater than 2 is unreliable, because too little light is reaching the detector to allow accurate measurement. Therefore, if the absorbance value is greater than 2, dilute the sample and repeat the measurement. Spectrophotometer is

a device that helps us to measure the amount of light reaching the detector. It contains a container called a cuvette, in which unknown sample is taken for measuring absorbance. 'Cuvettes' are usually a square object 1 cm across (as shown in Fig. 1.5). In some cases this container may not be square; however, one side of the vessel dimension is 1 cm and should be aligned with the light path. Some cuvettes are designed for visible light only, but usually a spectrophotometer is set for ultraviolet (UV) wavelength having wavelength below 340 nm (nanometer = 10^{-9} m = 1/1,000,000,000 n).

The term "spectroscopy" comes from the word "spectrum" which originally refers to the multiple colours of light apparent in an analysis of light using a prism. "Spectroscopy" therefore, implies the use of multiple wavelength of light. Spectrometer is a device to measure absorbance at a specific wavelength. It consists of a "monochromator" that splits the incidental light into its component wavelength and allows only light of the desired wavelength to reach the sample. This splitting of light is brought about either by a prism or by a diffraction grating. As extinction coefficient of a compound varies with wavelength, we can measure the absorbance at different wavelengths. The absorbance spectrum of a compound can vary depending on the chemical composition of the compound and depending on the solvent around the compound, i.e. the environment.

For example, protein has a strong (maximum) absorbance peak near 280 nm, but has very low absorbance at longer wavelengths (Fig. 1.6). The only chromophores (chemical groups within a compound that absorb light) in this protein the aromatic amino acids tryptophane and tyrosine are situated. For many proteins, these two are the only chromophores, which absorb only in the UV range, as they are colourless molecules. However, coloured proteins, such as haemoglobin (exhibit colour due to chromophore—Heme), absorb light in the visible portion of the spectrum.

The extinction coefficient of a molecule at a given wavelength can be calculated using the Beer-Lambert equation from absorbance measurement for solutions of known concentration.

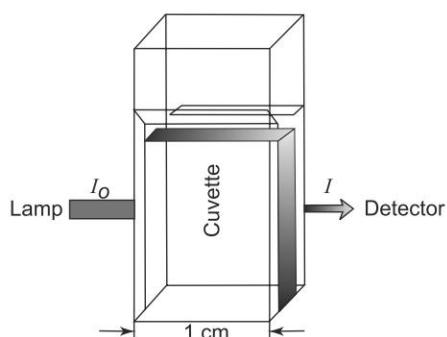


Fig. 1.5 A typical diagrammatic figure of an internal light transmission in a Spectrophotometer

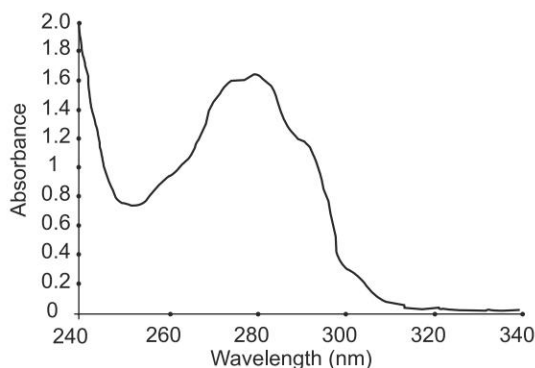


Fig. 1.6 Absorbance graph of a protein

Precautions

- Hold only the frosted sides of the cuvette.
- Make sure that your cuvette does not have a large absorbance when it contains only water.
- Solution should not have any artifacts, viz. fingerprints on the optical sides of the cuvette or objects floating in the solution or air bubbles, these artifacts will affect the absorbance.

(c) Preparing Dilutions

In biochemical analysis, molecular biology and plant tissue cultures, many solutions used are prepared by the dilution of more concentrated stock solutions. For preparing series of dilutions, you need to consider the goal of the procedure. You need to know both the desired final concentration and required volume of the diluted material. From the following simple equation, we can calculate readily:

$$C_1 V_1 = C_2 V_2$$

where, C_1 is the concentration of the initial (or stock) solution, V_1 is the volume of the initial solution available to be used for dilution, C_2 is the desired final concentration, and V_2 is the desired final volume. In most of the cases, at least three of the required terms are either known or can be chosen or decided by the experimenter.

Let us consider an example: You want to set up a standard curve. You have a stock solution of 1000 µg/ml bovine serum albumin (BSA) and for one of the points on the curve, you want 200 µg/ml. In this case, $C_1 = 1000$ µg/ml; $C_2 = 200$ µg/ml and $V_2 = 200$ µl. You need to know V_1 , i.e. how much of the stock solution must be diluted to 200 µl final volume to yield the desired concentration. By rearranging the dilution equation, we get

$$V_1 = V_2 (C_2 / C_1), \text{ and therefore } = 200 \text{ µl } (200 \text{ µg per ml} / 1000 \text{ µg per ml}) = 4 \text{ µl}$$

Thus, you need to dilute 4 µl of the stock solution to a final volume of 200 µl (i.e. by adding 196 µl).

Now, for example, you wish to make a solution of 1 µg/ml. The same equation will indicate that you need 0.2 µl of the 1000 µg/ml stock solution for 200 µl of the final diluted sample. Measuring 0.2 µl accurately is very difficult. If you do not have a Pipetman of the desired range, you have two choices: change the final volume (i.e. if V_2 is large, then V_1 must also increase), or perform serial dilution (i.e. instead of diluting the stock solution by a factor of 1000 in one step, dilute the stock solution and then make a further dilution of the diluted stock).

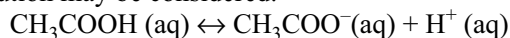
(d) Buffer and pH Determination

Most of the molecular genetics and biotechnological experiments deal with proteins and enzymes. These compounds are quite sensitive to changes in the concentration of various solution components. A buffer is a solution that is used to control the properties of a process occurring in an experimental aqueous medium. Buffer has the ability to resist changes in the hydrogen ion concentration of these solutions. In addition, it has also other molecules which influence the ionic strength, the activity of proteases and other

12 Analytical Techniques in Biotechnology

parameters of the experiments. There may be a slight change which is negligible and is taken as practically no change.

To understand the buffer action, a buffer solution prepared by mixing equimolar quantities of CH_3COOH and CH_3COONa in a common solution may be considered.



$$\begin{aligned} (\text{diss. const.}) k_D &= \frac{[\text{CH}_3\text{COO}^-][\text{H}^+]}{[\text{CH}_3\text{COOH}]} \\ &= 1.8 \times 10^{-5} \text{ (known)} \end{aligned}$$

$$\text{or} \quad [\text{H}^+] = k_D \frac{[\text{CH}_3\text{COOH}]}{[\text{CH}_3\text{COO}^-]}$$

and pH of the solution will be:

$$\text{pH} = -\log [\text{H}^+] = -\left(\log k_D + \log \frac{[\text{CH}_3\text{COOH}]}{[\text{CH}_3\text{COO}^-]} \right)$$

It is known that in equimolar solution:

$$\frac{[\text{CH}_3\text{COOH}]}{[\text{CH}_3\text{COO}^-]} = 1$$

$$\begin{aligned} \text{So,} \quad \text{pH} &= -\log k_D = -\log (1.8 \times 10^{-5}) \\ &= -(0.2552 - 5) = 4.7448 \end{aligned}$$

This pH of 4.74 will not be affected much by dilution or by adding H^+ or OH^- ions to it.

1. If some water is added the concentration of CH_3COOH and CH_3COONa giving CH_3COO^- ions will be affected equally such that the ratio $[\text{CH}_3\text{COOH}/\text{CH}_3\text{COO}^-]$ will be again unity, so pH will remain unchanged.
2. If a small amount of any acid $[\text{H}^+]$ ion is added, CH_3COO^- will react to produce CH_3COOH but as acetate is taken away, more of CH_3COONa will ionize, so again the ratio of $\text{CH}_3\text{COOH}/\text{CH}_3\text{COO}^-$ will become 1.
3. In the same way if a base, i.e. $[\text{OH}^-]$ ion is added, CH_3COOH in equilibrium will ionize to give a little more of CH_3COO^- ions but this change is negligible and the ratio $\text{CH}_3\text{COOH}/\text{CH}_3\text{COO}^-$ will remain 1.

Determination of pH Values Measurement and control of H^+ ion concentration are of great importance in every field. It varies over a wide range of say 1 in molar HCl solution down to 10^{-7} in pure water and 10^{-14} . In 1M NaOH solution at 25°C . A convenient scale for expressing H^+ ion concentration was introduced by Sorenson (1909).

$$\text{pH} = -\log [\text{H}^+] = \log \frac{1}{[\text{H}^+]}$$

Normally pH buffer solutions are determined with the help of a Potentiometer assembly and different electrodes, e.g. hydrogen, calomel or platinum electrode, etc. but for an accuracy of 0.02 pH units, the electrodes' potential must be measured to better than 1 mv, with high resistance glass electrode involved in the cell, the current should be 10^{-12} A or less. Now, a simple potentiometer cannot be used to measure the electromotive force (emf) of such a cell, because the instrument used for detecting the balance point of the potentiometer will not be sensitive to 10^{-12} A. So, for such measurement the electron tube Voltmeter is of special use. An ordinary potentiometer in conjunction with such a valve voltmeter can be used for working with the glass electrode. Normally, a potentiometer and valve voltmeter is combined into a single unit, the dials are then graduated in terms of pH, a rheostat is provided for balancing out the symmetry potential of the glass electrode. Such an instrument is known as a *pH-meter* (Fig. 1.7).

Calibration of a pH-meter Since different glass electrodes have different asymmetry potentials, it is necessary to calibrate the pH-meter with a buffer solution of known pH close to that of the test solution. Once the instrument is adjusted to give the known pH of the buffer solution, pH of other solutions can be obtained directly without any calculation.

(e) Metric Length and Fluid Volume

The study of microorganisms necessitates an understanding of the metric system of length. The basic unit of length is the meter (m). The basic unit

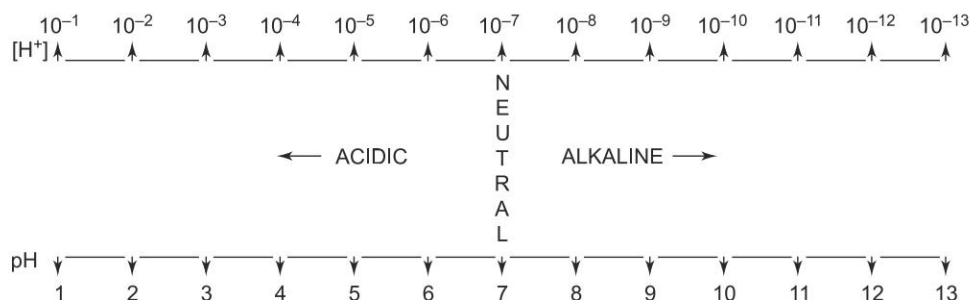


Fig. I.7 pH scale

for fluid volume is the litre (l). The prefix placed in front of the basic unit indicates fraction or multiple of that unit. The most common prefixes we will be using are:

Length: $1 \text{ m} = 10^3 \text{ mm} = 10^6 \mu\text{m} = 10^9 \text{ nm}$
 $= 10^{10} \text{ \AA}$

Weight: $1 \text{ g} = 10^3 \text{ mg} = 10^6 \mu\text{g}$

Volume: $1 \text{ l} = 10^3 \text{ ml} = 10^6 \mu\text{l}$

centi = 10^{-2} or 1/100

centimetre (cm) = 10^{-2} m or 1/100 m

milli = 10^{-3} or 1/1000 m

millimetre (mm) = 10^{-3} m or 1/1000 m

millilitre (ml) = 10^{-3} l or 1/1000 l

micro = 10^{-6} or 1/1,000,000

micrometre (μm) = 10^{-6} m or 1/1,000,000 m

microlitre (μl) = 10^{-6} l or 1/1,000,000 l

nano = 10^{-9} or 1/1,000,000,000

nanometre (nm) = 10^{-9} m or 1/1,000,000,000 m

In microbiology, we deal with extremely small units of metric length (micrometre, nanometre). The main unit of length is the micrometre (μm) which is 10^{-6} (1/1,000,000) of a metre.

The average size of a rod-shaped (cylindrical) bacterium is $0.05\text{--}1.0 \mu\text{m}$ wide by $1.0\text{--}4.0 \mu\text{m}$ long. An average coccus-shaped (spherical) bacterium is about $0.5\text{--}1.0 \mu\text{m}$ in diameter. A volume of one cubic inch is sufficient to contain approximately nine trillion average-sized bacteria. It would take over 18,000,000 average-sized cocci lined up edge-to-edge to span the diameter of a dime.

Viruses are usually much smaller than bacteria. Most are sub-microscopic, ranging from $10\text{--}250 \text{ nm}$.

Measurements commonly used to describe the length of DNA/RNA molecules are:

Base pair(s) or bp. = One bp corresponds to ca $3.4 \text{ \AA}$ of length along the strand.

Kilo base pairs (Kb) = 1000 bp

Mega base pairs (Mb) = 1,000,000 bp

Giga base pairs (Gb) = 1,000,000,000 bp.

In case of single strands DNA/RNA we talk about nucleotides, abbreviated as nt (or Knt, Mnt, Gnt) rather than base pairs, as because they are paired.

(f) Using a Microscope

There are different types of microscopes, viz. Dissecting, Compound, Fluorescence, Phase Contrast and electron microscopes. A *Compound microscope* is one of the most important and useful tools in biological studies. It is used to study cells, cell parts and many other important aspects. As many of the experiments will require a compound microscope, it is important to get familiar with its parts, its uses and care.

Moving and Transporting the Microscope

Grasp the arm of the microscope with one hand and support the base of the microscope with the other. Handle the microscope gently.

Adjusting the Eyepieces These microscopes are either mono- or binocular, i.e. they have one or two ocular lenses (eyepieces). To adjust them, first find the proper distance between your eyes and the eyepieces by closing one eye and slowly moving your head towards that eyepiece until

you see the complete field of view—about 1 inch away. Keep your head steady and both eyes in the same plane. Now open the other eye and gradually increase the distance between the eyepieces until it matches the distance between your eyes. At the correct distance you will see one circular field of view with both eyes.

Positioning the Slide Place the slide specimen side-up on the stage so that the specimen lies over the opening for the light in the middle of the stage. Secure the slide between and not under the slide holder arms and the mechanical stage. The slide can now be moved from place to place using the 2 control knobs under the stage on the right of the microscope.

Adjusting the Illumination The mirror is adjusted (or voltage control dial) to obtain a clear and bright circular microscopic field. For oil immersion microscopes (1000X) you will need an artificial source of light.

At lower magnification, less light is needed. Adjust the amount of light coming through the condenser using the iris diaphragm lever located under the stage in front of the microscope. Light adjustment using the iris diaphragm lever is critical for obtaining proper contrast. For oil immersion microscopes (1000X), the iris diaphragm lever should be set almost all the way open (for maximum light). For low powers such as 100X the iris diaphragm lever should be set mostly closed (for minimum light). The condenser height control, i.e. the single knob under the stage on the left-hand side of the microscope should be set so that the condenser is all the way up.

Adjusting the Magnifications The final magnification is a product of the two lenses used. The eyepiece or ocular lens magnifies 5X, 10X or 15X. The objective lenses are mounted on a turret near the stage. The smaller lenses magnify 10X or 40X and oil immersion lens magnify 100X. All the three lenses are fitted on a slip nose. The final magnifications are as follows:

Ocular lens (eye piece)	X	Objec- tive lens	=	Total magnification
5X	X	10X	=	50X
10X	X	10X	=	100X
10X	X	40X	=	400X
10X	X	100X	=	1000X
		(oilimm- ersion)		

Reason for Using Immersion Oil Normally, when light waves travel from one medium into another, they bend. Therefore, as the light travels from slide to air, the light waves bend and are scattered similar to the “bent pencil” effect when a pencil is placed in a glass of water. The microscope magnifies this distortion effect. Also, if high magnification is to be used, more light is needed.

Immersion oil has the same refractive index as glass and therefore, provides an optically homogenous path between the slide and the lens of the objective. Light waves thus travel from the glass, into glass-like oil, into the glass lens without being scattered or distorting the image. In other words—the immersion oil “traps” the light and prevents the distortion effect that is seen as a result of the bending of the light waves

Focusing from Lower Power to Higher Power

Follow the following steps:

1. Rotate the 10X objective until it locks into place (total magnification of 100X).
2. Turn the coarse focus control (large knob) all the way away from you until it stops.
3. Look through the eyepieces and turn the coarse focus control (large knob) towards you slowly until the specimen comes into focus.
4. Get the specimen into sharp focus using the fine focus control (smaller knob) and adjust the light for optimum contrast using the iris diaphragm lever.
5. If higher magnification is desired, simply rotate the 40X objective into place

(magnification 400X) and the specimen should still be in focus (fine focus and light contrast may be needed).

6. For maximum magnification (1000X or oil immersion) rotate the 40X objective slightly out of position and place a drop of immersion oil on the slid. Now rotate the 100X oil immersion objective into place. Again, the specimen should remain in focus, although minor adjustments in fine focus and light contrast may be needed.

Cleaning the Microscope Clean the exterior lenses of the eyepiece and objective before and after the observation using lens paper only. Remove any immersion oil from the oil immersion lens before putting the microscope away. You may use pure silk cloth and Xylene for cleaning lenses and immersion oil.

(g) Binomial Nomenclature

Microorganisms are given specific scientific names based on the binomial (two names) system of nomenclature. The first name is referred to as *genus* and the second name is termed *species*. The name usually come from Latin or Greek and describes some characteristic of the organism incorporates the name of the discoverer.

To correctly write the scientific name of a plant or microorganism, the first letter of the genus should be capitalized while the species name should be in smaller letters. Both the genus and species names are italicized or underlined. Some examples are given below:

- *Bacillus subtilis* or Bacillus subtilis
Bacillus: L. dim. noun *Bacillum*, a small rod
subtilis: L. adj. *subtilis*, slender
- *Escherichia coli* or Escherichia coli
Escherichia: after discoverer, Prof. Escherich
coli: L. gen. noun *coli*, of the colon
- *Staphylococcus aureus* or Staphylococcus aureus
Staphylococcus: Gr.noun *Staphyle*, a bunch of grapes; Gr. noun
coccus, berry
aureus: L.. adj. *aureus*, golden

(h) Centrifugation Technique

Centrifugation is a process used to separate or concentrate materials suspended in a liquid medium. The theoretical basis of this technique is the effect of gravity on particles (including macromolecules) in suspension. Two particles of different masses will settle in a tube at different rates in response to gravity. Centrifugal force (measured as xg, gravity) is used to increase this settling rate in an instrument called a centrifuge.

Suitable Rotor A centrifuge machine is a manually or digitally controlled device which has a motor for spinning the rotor, a break and a timer. Sometimes the instruments (e.g. in refrigerated centrifuge system) also contains a temperature adjustable mode switch. Rotors for a centrifuge are either fixed angles, swinging buckets, continuous flow, or zonal, depending upon whether the sample is held at a given angle to the rotation plane, is allowed to swing out on a pivot and into the plane of rotation, designed with inlet and outlet ports for separation of large volumes, or a combination of these.

Centrifuge Tubes of Suitable Size which can Fit Appropriately into the Rotor

For biological work, the tubes are divided into functional groups, made of regular glass, Corex glass, nitrocellulose, or polyallomer. Regular glass centrifuge tubes can be used at speeds below 3,000 rpm that is in a standard clinical centrifuge. For work in the higher speed ranges, centrifuge tubes are made of plastic or nitrocellulose. Preparative centrifuge tubes are made of polypropylene (sometimes polyethylene) and can withstand speeds up to 20,000 rpm.

Calculation Relative Centrifugal Force

The centrifugal force is dependent upon the radius of the rotation of the rotor, the speed at which it rotates, and the design of the rotor itself (fixed angle, vs swinging bucket). Rotor speed and design can be held constant, but the radius will vary from the top of a centrifuge tube to the bottom. If a measurement for the radius is taken as the mid-point, or as an average radius, and all forces are mathematically related to gravity, then

one obtains a relative centrifugal force, labelled as xg . Centrifugation procedures are given as xg measures, since RPM and other parameters will vary with the particular instrument and rotor used. Relative Centrifugal Force (RCF) is a constant that is independent of the apparatus used.

A simple formula for calculating this value is:

$$\text{RCF} = 1.12 r (\text{rpm}/1000)^2$$

where, r = radius in millimetres.

The difficulty with using the formula is establishing the value for r . Typically, there are three r values given (by the manufacturer) for a rotor: the maximum, minimum and average r . These correspond to the distances from the centre of rotation to the bottom, top and middle of the sample tube.

Procedure

1. The material to be centrifuged should contain a solute and a solvent; or two liquids of different density gradients. If one aims to obtain different compounds from a solid matter, then one should first crush the solid material using a suitable buffer or solvent.
2. The material to be “spun” is placed in a centrifuge tube which is then placed in a rotor. The tubes should be placed in opposite slots and balanced. The rotor is generally a dense metal which dissipates heat quickly, and is of sufficient mass that it generates momentum, i.e. once it is spinning it requires little energy to keep it going. Centrifuges generally work under vacuum and are refrigerated to reduce heating caused by frictional forces as the rotor spins. Rotors are usually stored in refrigeration units to keep them at or near the operating temperature.
3. If the density and viscosity of the medium, as well as the density of a given particle, are known, then the time needed to completely sediment a particle can be determined by the formula:

$$T = \{(D - L)/(D + L)\} * (N) / \{d^2(g - p)S^2\}$$

where, T = time in minutes; D = radial distance in cm for r_{max} ; L = radial distance to meniscus;

N = viscosity of the fluid medium; g = density of the fluid medium; p = density of the particle to sediment; d = diameter of the particle in cm; and S = rotational velocity in rpm.

4. After centrifugation time is over and the rotors come to a complete standstill the tubes should be taken out slowly and carefully. One can observe the separation, sedimentation or precipitation of materials based on their increasing density gradient from top to bottom. One can now collect the differentiated layers in separate tubes and follow the recommended steps for its processing; or may carefully throw out the supernatant and use the precipitate for further processing as recommended by the experimental procedure.

Precautions

Before running a centrifuge, check the classification decal on the centrifuge to ensure that the rotor is safe to use in the centrifuge at hand.

- Never use an alkali detergent on a rotor (most are highly alkaline—be sure to check before use).
- Always clean and completely dry the rotor after every use. Any spilled materials, especially salts and corrosive solvents, must be removed immediately with running water. Fixed angle rotors are stored upside down, to drain after thorough cleaning and rinsing. Swinging buckets have only the buckets cleaned and dried, and stored inverted and with the caps removed. NEVER immerse the rotor portion of a swinging bucket rotor. Inevitably the linkage pins will rust, as it is virtually impossible to remove all fluids from them.
- Be especially careful not to scratch the surface of a rotor or bucket. Use plastic brushes only. Normal wire brushes will scratch the anodized surface of aluminium rotors which will increase the likelihood of corrosion. The anodized layer is extremely thin and is the main defence against corrosion of an aluminium rotor.
- Always use the proper centrifuge tube.

- Always fill the centrifuge tubes to the proper level (usually full to within $\frac{1}{2}$ inch of the top). The tubes are thin walled and will collapse if improperly filled.
- Always balance the rotor properly. Use a precision scale for most work. Always balance the tube with a medium that is identical to that being centrifuged, i.e. do not balance an alcohol solution with water, or a dense sucrose solution with water only. For swinging buckets, be sure that the buckets are weighed with their caps in place, that the seals are intact and that the caps are secure. Be careful in the placement of tubes within a rotor to ensure proper balance—check the manufacturer's guide for complex rotors that hold multiple tubes.
- Ensure that the rotor is properly seated within the centrifuge. For swinging buckets, ensure that they are hanging properly—*Double or triple check!* For preparative rotors, be sure the rotor cover is in place and properly screwed down, where appropriate. *NEVER* use a rotor without its lid, when one is supplied—the screw actually holds the rotor to the motor shaft.
- Check that the centrifuge chamber is clean, defrosted and that all membranes or measuring devices are in tact and functional (Beckman speed and temperature controls) and that the lid is securely closed.
- Adjust acceleration rates, deceleration rates, temperature and rpm controls as appropriate. Set brake on or off as appropriate and check vacuum level where appropriate.
- Start the centrifuge and set the timer. Do not attempt to open the centrifuge until the rotor has come to a complete stop.
- Before opening the centrifuge, record the appropriate information in the centrifuge log book.

(i) Polymerase Chain Reaction (PCR)

In a short time since its discovery by Kary Mullis (1985), PCR has revolutionized the approach to molecular biology. The impact of PCR on biologi-

cal and medical research is immense. A PCR is commonly called a 'DNA photocopier'. PCR uses a few basic components to make large number of copies of a specific DNA fragment in a tube.

PCR uses only the basic components of the complex replication machinery to replicate short fragments of DNA in a tube. In a simple buffer system, a region of interest of a template DNA molecule is copied by a DNA polymerase (e.g. Taq DNA polymerase) that uses deoxynucleotides as building blocks of the new strands. Sequence specific oligonucleotide primers that bind to the template according to normal base pairing rule (A-T, G-C) define the region to be copied. The strands of the template are separated by heat, which causes the breakage of hydrogen bonds between the base pairs of a DNA molecule, in a process called *denaturation*. The oligonucleotide primers then find their complementary sequences on the template (annealing) and DNA polymerase then begins to add deoxynucleotides to the 3'-OH group of the primers, producing new duplex molecules. In the next step, these new double stranded DNA molecule act as template while repeating the whole replication and synthesis machinery. There is an exponential increase in the number of 'copies' of the 'target DNA' (Fig. 1.8; page 19).

Setting of PCR Conditions

Switch on the PCR machine, and by following the steps recommended by the manufacturer set the PCR temperature and the cycling condition, e.g. we can set up the conditions for a standard PCR reaction as shown in illustration on next page.

It has to be noted that the annealing temperature for a PCR is determined based on the average T_m of the primers (forward and reverse) used in the PCR reaction. The T_m of a specific primer (forward or reverse) can be calculated as: $T_m = 2(A + T) + 4(G + C)$. The best annealing temperature has to be optimized and determined by playing around with the average $T_m \pm 4^\circ\text{C}$.

Setting of a PCR Reaction Mixture

1. The constituents except (Taq polymerase) should be thawed on ice before setting up the reaction mixture.

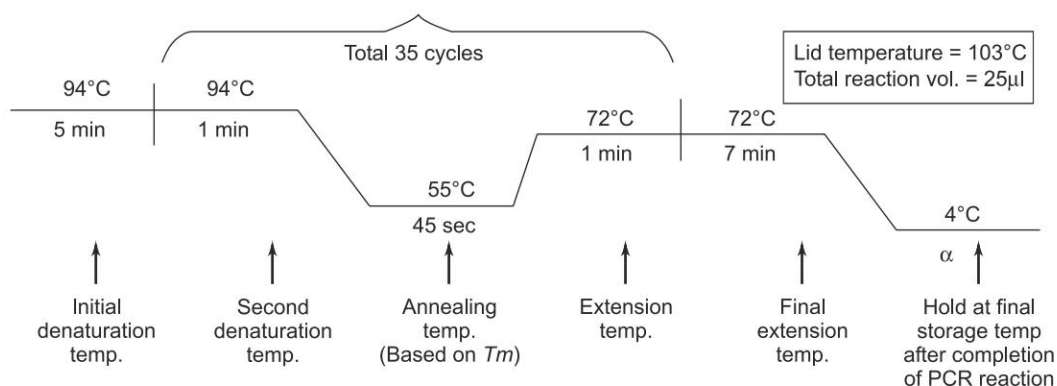


Fig. 1.8 Setting of PCR Conditions

2. A standard 25 µl reaction mixture may be formulated as following in a PCR tube:

Nuclease free water	– 18.4 µl
10X PCR buffer	– 2.5 µl
25 mM MgCl ₂	– 1.5 µl
10 mM dNTP mix	– 0.5 µl
10 pmoles/ µl Primer F	– 0.5 µl
10 pmoles/ µl Primer R	– 0.5 µl
5 U/µl Taq DNA polymerase	– 0.1 µl
	(should be added last)
Template	– 1.0 µl
	(~50 ng if quantified earlier)

One should note that the amount of MgCl₂ to be used has to be experimentally optimized by setting of PCR separate reactions using various concentration of the MgCl₂.

Starting the PCR

- After preparation of the reaction mixture is ready, check the lid temperature of the PCR (which should be > denaturing temperature, usually 102–103 °C).
- Open the lid and put the PCR tube in the slot in the base plate provided inside. Close the lid and click the option Start. Insert the volume amount, i.e. 25 µl if asked by the PCR programme.
- Let the PCR machine perform the reaction and store back all the reagents at –20 °C.

Post-PCR Analysis Once the PCR is over, you need to check or analyze the products. The success or failure of a PCR is determined by three important features:

- Is there a single product band?
- Is it of the expected size?
- Are there other products and is the major one your target?

Depending on the success of the PCR reaction, it may be necessary to optimize the conditions to achieve improved results. (Analysis of the results can be done either by native gel electrophoresis or by agarose gel electrophoresis which are discussed in detail in the chapter on Electrophoresis.

Precautions

As PCR is a very sensitive experimentation procedure, one has to be very careful while setting up of the experiment till analysis. Some precautions to be followed are:

- All the chemicals should be properly **stored at –20 °C deep freezer, and should not be cross-contaminated**. Preferably the constituents may be aliquoted in separate vials to be used by separate groups of students.
- The pipettes used for setting up the reaction should be clean and well calibrated.
- All the tips and vials used in the PCR reaction should be properly autoclaved before use.
- One should put the PCR reaction in a separate clean room, and should preferably wear gloves to avoid contamination.

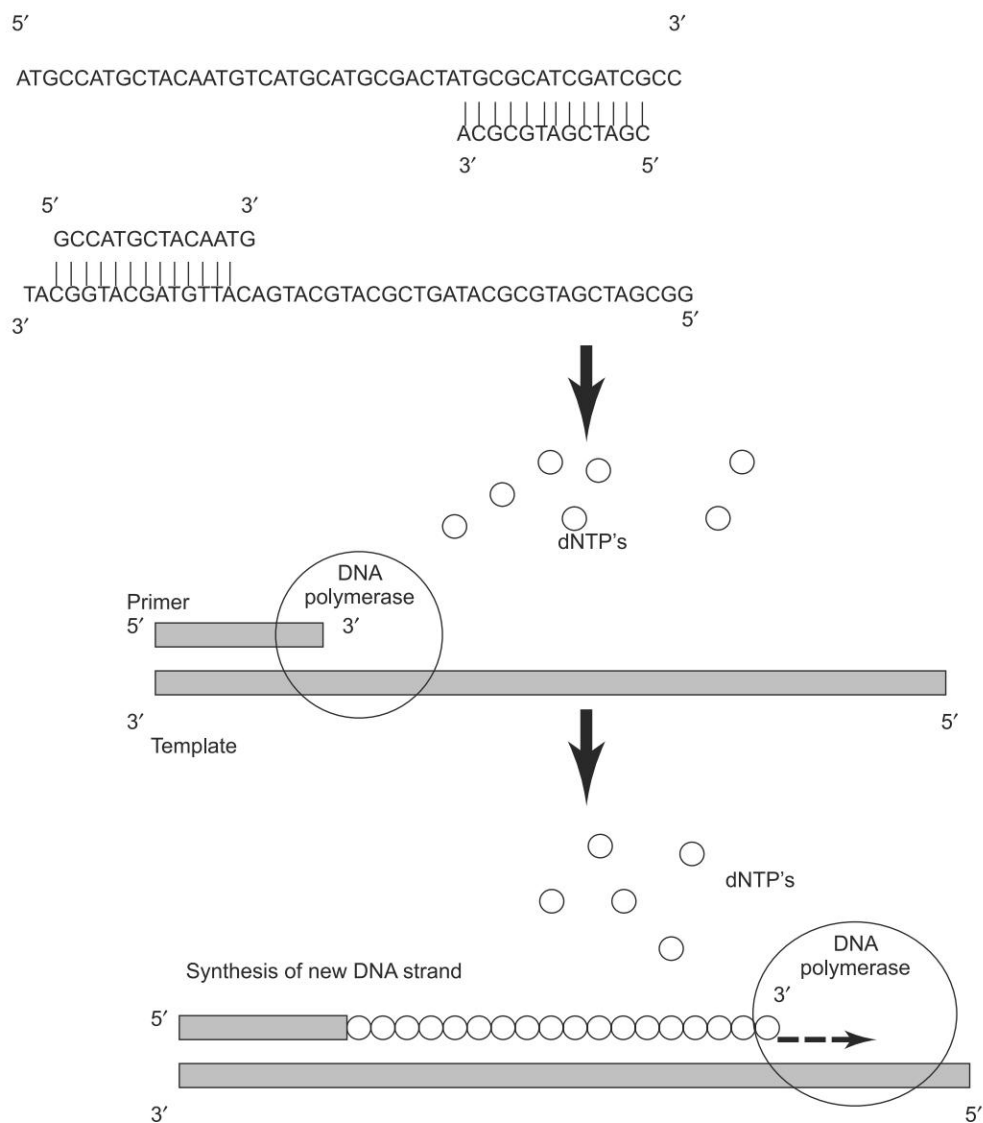


Fig. 1.9 Diagrammatic representation of PCR showing primer extension and synthesis of new DNA strand by DNA polymerase. The primer anneals to a complementary sequence on the template strand and the DNA polymerase uses the template sequence to extend the primer by incorporation of the correct deoxynucleotide (dNTP)

- The PCR machine should be linked to a UPS backup for uninterrupted power supply.
- The buffer to be used for agarose gel electrophoresis should be autoclaved and nuclease free.
- A positive (PCR reaction with a standard template) and a negative control (PCR reaction without template) should be always used when conducting a PCR reaction experiment.
- The PCR reactions and all the events should be conducted in a dust-free room.
- Switch on and off the machine as per your necessity to use the machine.

(j) Electrophoresis Technique

Electrophoresis is a technique for separating or resolving molecules in a mixture under the influence of an applied electric field, also called electrophoretic mobility. Dissolved molecules in an electric field move, or migrate at a speed determined by their charge : mass ratio. For example, if two molecules have the same mass and shape, the one with the greater net charge will move faster toward an electrode.

Electrophoresis may be the main technique for molecular separation in today's cell biology laboratory. It has become very common because it is such a powerful technique, and yet reasonably easy as well as inexpensive. In spite of the many physical arrangements for the apparatus, and regardless of the medium through which molecules are allowed to migrate, all electrophoretic separations depend upon the charge distribution of the molecules being separated. The separation of small molecules, such as amino acids and nucleotides, is one of the many uses of electrophoresis. In this case, a small drop of sample is deposited on a strip of filter paper or other porous substrate, which is soaked with a conducting solution. When an electric field is applied at the ends of the strip, small molecules dissolved in the conducting solution move along the strip at a rate corresponding to their magnitude of their charge.

Electrophoresis can be one-dimensional (i.e. one plane of separation) or two-dimensional. One-dimensional electrophoresis is used for most routine protein and nucleic acid separations. Two-dimensional separation of proteins is used for finger-printing, and when properly constructed can be extremely accurate in resolving all of the proteins present within a cell.

(i) Agarose Gel Electrophoresis (Basic Method)

Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA. The purpose of the gel might be to look at the DNA, to quantify it or to isolate a particular band. The DNA is visualized in the gel by addition of ethidium bromide (EtBr). This binds strongly to DNA by intercalating between the bases and is

fluorescent, i.e. it absorbs invisible UV light and transmits the energy as visible orange light.

Most agarose gels are made between 0.7% and 2%. A 0.7% gel will show good separation (resolution) of large DNA fragments (5–10 kb) and a 2% gel will show good resolution for small fragments (0.2–1 kb). Some people go as high as 3 to 4% for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case. Low percentage gels are very weak and may break when you try to lift them. High percentage gels are often brittle and do not set evenly.

Recipe for 2 l of 10x TBE:

218 g Tris base
110 g Boric acid
9.3 g EDTA

Dissolve the ingredients in 1.9 L of distilled water. pH to about 8.3 using NaOH and make up to 2 L.

50X TAE Buffer:

242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5M EDTA (pH 8.0)
Bring up to a litre with dH₂O

Procedure

1. Weigh out 1 g of analytical or molecular biology grade agarose.
2. Add to 100 ml of autoclaved 1x TAE/TBE (see above for 50X solution)
3. Microwave the solution in order to dissolve the agarose.
4. Let it cool to 60°C (at this point ~1–2 µl of 10 mg/ml EtBr may be added: soaking the gel in EtBr later will not be necessary)
5. Pour it into the electrophoresis apparatus
6. After the gel has solidified, pour the 1x TAE/TBE into the gel box and remove the comb. Samples are loaded into the gel only after the buffer solution is in the gel box covering the gel.
7. Load the samples to be checked by electrophoresis on to respective wells (No. 2 onwards) after mixing with a loading dye. Load the standard ladder in well No. 1.

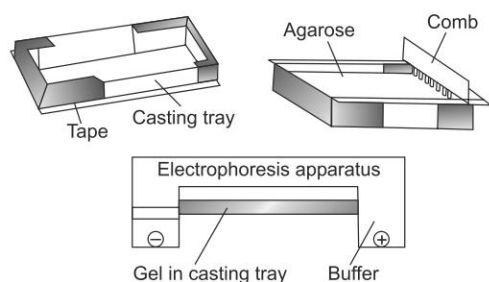


Fig. 1.10 Electrophoresis apparatus

8. Set the parameters of current (~ 400 mA) and voltage (~ 80 volts), and run the gel till almost the dye front has reached $\frac{3}{4}$ of the gel.
9. Dip the gel in EtBr solution for 5 min (if EtBr was not added earlier) and visualize the gel on a transilluminator or gel doc system (if EtBr is previously added, then the gel may be checked directly under UV).
10. Note down the size of the expected band by comparing with the standard marker's size.
11. Take a photograph of the agarose gel and paste it in your practical notebook.

Precautions

- The agarose gel should be completely submerged in the buffer system.
- The buffer should be routinely changed.
- Do not put your hands in the buffer, or touch the gel with bare hands while handling it as it contains EtBr, which is carcinogenic.
- The gel should be properly discarded after use.

(ii) SDS-Polyacrylamide Gel Electrophoresis

1. Electrophoretic separation of proteins is most commonly performed in polyacrylamide gels. These gels are cast between a pair of glass plates by polymerizing a solution of acrylamide monomers into polyacrylamide chains and simultaneously crosslinking the chains into a semisolid matrix. The pore size of a gel can be varied by adjusting the concentration of polyacrylamide and the crosslinking reagent.
2. Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide

and N,N-methylene-bis-acrylamide (Bis, for short). Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium persulphate along with N,N,N,N,-tetramethylethylenediamine (TEMED). The gels are neutral, hydrophilic, three-dimensional networks of long hydrocarbons cross-linked by methylene groups.

3. The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The pore size of a gel is determined by two factors, the total amount of acrylamide present (designated as %T) and the amount of cross-linker (%C). As the total amount of acrylamide increases, the pore size decreases. With crosslinking, 5 % C gives the smaller pore size. Any increase or decrease in %C increases the pore size. Gels are designated as percent solutions and will have two necessary parameters. The total acrylamide is given as a % (w/v) of the acrylamide plus the bis-acrylamide. Thus, a $7\frac{1}{2}$ %T would indicate that there is a total of 7.5 g of acrylamide and Bis per 100 ml of gel. A gel designated as 7.5%T: 5%C would have a total of 7.5% (w/v) acrylamide + Bis, and the Bis would be 5% of the total (with pure acrylamide composing the remaining 2.5%).
4. When a mixture of proteins is applied to a gel and an electric current is applied, smaller proteins migrate faster than larger proteins through the gel. The rate of movement is influenced by the gel's pore size and the strength of the electric field. The pores in a highly cross-linked polyacrylamide gel are quite small. Such a gel could resolve small proteins and peptides, but large proteins would not be able to move through it.

Role of SDS in SDS-PAGE Gel Electrophoresis

- Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used in biochemistry, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight as well as higher order protein

folding, post-translational modifications and other factors). This technique can be used to determine whether a given protein is present in a sample, and for assessment of purity of the preparation, for estimation of the approximate quantity of the protein, and for measurement of the size of the protein.

Electrophoresis is a process in which molecules are exposed to an electric field and separated on the basis of their differential mobility's in that field. The observed differential mobility is a result of different charge magnitudes on different molecules, and the result of different resistance to movement through the medium.

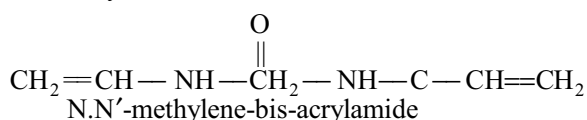
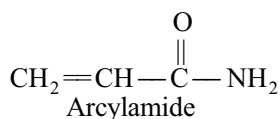
The velocity of a charged molecule is given by:

$$v = \frac{qE}{f}$$

where, q is the charge on the molecule, E is the electrical potential gradient, and f is the frictional coefficient of the medium for the molecule. For similarly shaped molecules, f is proportional to size of the molecule. For molecules in which charge increases in proportion to size, larger molecules move more slowly than small ones, because f increases faster than charge.

- Gel electrophoresis uses a matrix of large uncharged molecules to provide the required friction. The matrix also serves to inhibit diffusion, and therefore to prevent degradation of the separation of proteins usually involves the use of polyacrylamide as the matrix.

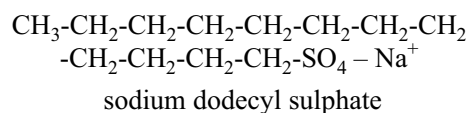
Polyacrylamide is formed by polymerization of acrylamide monomers in the presence of N,N'-methylene-bis-acrylamide.



The bis-acrylamide contains two double bonds, which allow the compound to act as a crosslinker

between polyacrylamide chains. The presence of the crosslinking agent results in formation of a gel matrix than a simple linear polymer. The polymerization reaction is a serial reaction using a free radical mechanism. The formation of the free radicals is initiated by the unstable compound ammonium persulfate. The sulphate radicals formed then react with tetramethylethylenediamine (TEMED), forming TEMED radicals which react with acrylamide to begin the actual polymerization reaction.

- The solution of protein to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non-disulfide-linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure. This is known as Native PAGE. Adding SDS solves this problem, as it binds to and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide.



- SDS bind in a ratio of approximately 1.4 g SDS per 1.0 g protein (although binding ratios can vary from 1.1–2.2 g SDS/g protein), giving an approximately uniform mass: charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein. A tracking dye may be added to the protein solution to allow the experimenter to track the progress of the protein solution through the gel during the electrophoretic run.
- As mentioned, the proteins are mixed with SDS, an anionic detergent which binds to the proteins and denatures their secondary and non-disulfide-linked tertiary structure with the addition of heat. SDS also applies a uniform negative electrical charge to each protein in proportion to its mass.

- If SDS is not used in separation, proteins with similar molecular weights would migrate differently in the gel due to differences in their mass to charge ratios. As proteins are separated using electrical current and pore size of the gel matrix, differences in charges (in addition to mass) would play a role in separation.

Table 1 List of approximate useful ranges for different gel densities:

PERCENT ACRYLAMIDE	USEFUL MOLECULAR WEIGHT RANGE DALTONS
7	30000 to 200000
10	20,000 to 150,000
12	10,000 to 100,000
15	5000 to 70,000

Procedure for Preparation of Stock Solutions

- 1.5 M Tris.HCl pH 8.3 + 0.4% SDS (adjust pH before you add the SDS). Keep at RT.
- 30% Acrylamide 0.8% Methylene bis Acrylamide or (ratio: 37.5:1). Keep at 4°C.
- 0.5M Tris.HCl pH 6.8 + 0.4% SDS (adjust pH before you add the SDS). Keep at RT.
- 10% Ammonium persulfate (APS). Keep at 4°C for less than 1 month.

A standard working volume of constituents to be used for mid-size SDS-PAGE electrophoresis was standardized and is tabulated below:

Separating gel

CONSTITUENTS	GEL PERCENTAGE			
	14%	12%	10%	8%
30% acrylamide	7 ml	6 ml	5 ml	4 ml
4X Tris (pH 8.8)	3.75 ml	3.75 ml	3.75 ml	3.75 ml
H ₂ O (MQ)	volume adjusted up to 15 ml			
10% APS	125 µl	125 µl	125 µl	125 µl
TEMED	12 µl	12 µl	12 µl	12 µl

Stacking gel

CONSTITUENTS	AMOUNT
30% acrylamide	0.65 ml
8X Tris.Cl/SDS (pH 6.8)	0.625 ml
H ₂ O (MQ)	Make volume up to 5 ml
10% APS	25 µl
TEMED	7 µl

Add TEMED and APS at the end. Gently swirl the flask to mix, being careful not to generate bubbles.

Dissolving (Sample) Buffer x5

Glycerol	5 ml
SDS	1 g
βMercapto ethanol (or 0.25M DTT)	2.56 ml
0.5M TrisHCl pH 6.8 + 0.4% SDS	2.13 ml
Bromo phenol blue	traces

Keep in aliquots of 1 ml at -20°C

Running Buffer 5X

Trizma	0.125 m	15.1 g
Glycine	0.96 m	72.0 g
SDS	0.5%	5.0 g
H ₂ O up to		1 l

Keep at RT

Staining Solution

Methanol CP	500 ml	50 %
Acetic acid CP	100 ml	10 %
H ₂ O	400 ml	
Coomassie Brilliant Blue R	2.5 g	0.25 %

Destaining Solution

Methanol CP	150 ml	15 %
Acetic Acid CP	100 ml	10 %
H ₂ O	750 ml	

Keep flask on dark at RT

Preparation of the gel and performing electrophoresis

1. Clean the pair of glass plates (or one glass plate and one silica plate) with tissue paper or cotton.
2. Insert the spacers of specific size at the sides and fix the plates on a gel caster or seal the lower side by agarose.
3. Prepare the separating gel of required concentration and pour quickly. Fill the maximum space between the plates leaving about 4 cm from the top.
4. Allow it to solidify by keeping it undisturbed for 15 minutes.
5. Prepare the stacking gel and pour on the top half of the gel plates above the separating gel layer and insert the combs (Fig. 1.9).
6. Prior to adding the sample buffer, keep samples at 0°C. Add the SDS sample buffer (RT) to the sample (still on ice), and boil at 100°C

24 Analytical Techniques in Biotechnology

immediately for 3 to 5 min. Once heated, sample could sit at RT or ice for a short time until loading, or at -20°C for a long time.

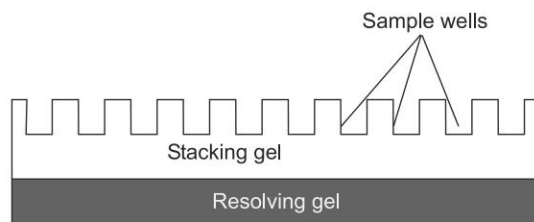


Fig. I.11 Diagrammatic representation of an SDS-PAGE gel, showing arrangement of stacking and separating gel and a typical band pattern in the gel plates

7. Take out the comb after the solidification of the stacking gel, and fix it to the vertical electrophoresis running apparatus.
8. Fill the tank with running buffer by diluting it to 1X.
9. Load the samples in respective wells and also a standard protein marker.
10. Connect the two electrodes of the gel apparatus to the power pack and adjust the voltage at 80 V and current at maximum. Let the electrophoresis to continue till the blue front of the dye in the samples moves out of the gel.
11. Dismantle the gel carefully out of the gel plates and put it in the staining solution.
12. Stain overnight at RT or put gel with a staining solution shaker, and then shake for another 15 min at RT. Wash with water 2–3 times and destain by several changes of destaining solution.

(**Note:** The gel percentage was used depending on the size of the protein whose differential expression profile was to be studied. The gel concentration and protein size are inversely correlated. It is best to use a concentration of between 15–18 μg for coomassie brilliant blue staining.)

The denatured proteins are differentially seen (in blue colour) as separate fragments depending on their molecular weight. You can note the number of bands of different molecular weight comparing with the standard protein marker. If

comparing between two samples for the expression of protein of a specific molecular weight, make an observation by selecting out the molecular weight of the specific protein you are looking for in the two samples to be compared, and analyze in which sample the intensity of the protein band is intense. Although it is not accurate to predict that only the protein of your interest in one sample is high, as many proteins may be of similar size, but it is a rough prediction and can be simultaneously proved by further experiments like Western blotting, etc.

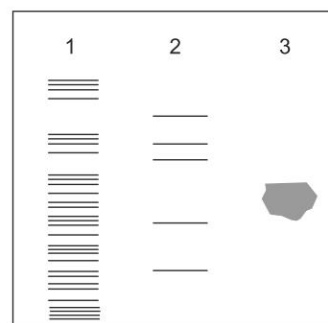


Fig. I.12 Diagrammatic gel showing the same mass of total protein loaded in each lane. 1st lane—Many proteins, none resulted in an intense band, 2nd lane—Showing band for five visible proteins, Correctly loaded, 3rd lane—Overloaded

Precautions

- One should wear gloves and mask while opening and preparing acrylamide solutions.
- The preparation and pouring of the separating and stacking gel should be done fast to prevent solidification.
- One should be careful using the glass and the silica plates.
- Quantification of proteins before loading is mandatory. This can be done using various biochemical methods like Lowry's and BCA method, etc.

Note: Electrophoretic techniques may be used for DNA sequencing, southern blotting, western blotting, etc. which are not within the purview of this manual.

(k) Sterilization Techniques

Maintaining of culture devoid of microorganisms is very important during the transfer, culture of microbes, plant and animal cells, as most of the nutrient media support the growth of bacterial, fungal and algal contaminants which spoils the culture. Another important thing is the sterilization of tools, surgical instruments, glass or plastic culture vessels, nutrient media and plant material. The common methods employed for these purposes may be discussed under the two following sub-headings, viz. (a) Plant tissue culture, and (b) Aseptic culture of microbes.

(a) Plant Tissue Culture

There are three different types of sterilization used in plant tissue culture:

(i) *Heat (Dry or Wet) Sterilization* Mainly high temperature or heat (ranging 80° to 160 °C for 2 h) is used for sterilization of washed glassware or surgical metallic tools are used for drying in oven. It can also be done by autoclaving (which functions like a pressure cooker). Generally, steam is generated in a tightly enclosed vessel to a temperature of 121 °C to develop 15 to 20 lb/in² pressure for 15 to 20 min. Materials like glassware, paper products, tissue culture tools (e.g. surgical blades, scalpels, inoculating needles, forceps), liquids (e.g. sterile water, plant tissue culture or microorganism culture broth in glassware, etc.) is generally wrapped in a heavy duty aluminium foil or thick paper. However, if paper is used for wrapping, all packing should be placed in the oven for a few min at 60°C after autoclaving for evaporation of moisture.

(ii) *Ultra-filtration Sterilization* There are many chemicals, e.g. enzymes, GA₃ and antibiotics which are unstable and heat-sensitive or heat-labile. Generally, membrane filters having pore sizes of 0.22, 0.45 and 0.55 mm are commercially available (e.g. Millipore and Whatman membrane filters). Filters are made up of cellulose acetate and/or cellulose nitrate sealed in a pre-sterilized container or plastic disposable unit or autoclavable units. Liquid culture media are passed through these

membrane-filters fitted in an enclosed chamber unit equipped with vacuum facility. Usually, 0.22 mm membrane-filters are common.

(iii) *Chemical Sterilization* In tissue culture experiments, plant materials, such as leaves shoot meristems, stems, internodes, seeds, tissue, etc. are surface sterilized.

- Plant parts are usually incubated in an aqueous solution of optimal concentrations of many disinfectants, viz. detergents, chlorax, mercuric chloride (HgCl₂), sodium hypochloride (NaClO), etc.
- Plant materials which are to be inoculated are dressed and trimmed to desirable size, then washed in a running tap water, imbibed in Tween-20 before sterilization in an appropriate chemical concentration and duration.
- After that they are thoroughly washed with sterile double distilled water several times (at least 4-5 times).
- Before inoculation, all materials, viz. plant parts, culture tubes or petri plates, forceps, scalpel, spirit lamp, etc. are placed inside the laminar air flow cabinet hood and then UV lamp (with wavelength of ~254 nm) should be put on for 30 min.
- Detergent, viz. Chlorax or Tween-20 and Tween-80 are also available. Commercially, 5 to 25 per cent sodium hypochloride is also available. Usually, 0.05% (V/V) detergents or 0.1 to 1.0% HgCl₂ are used for 1 to 10 min for surface sterilization.
- Absolute alcohol or 70% ethyl alcohol (C₂H₅OH) are used as disinfectant. For seeds with hard seed coats, e.g. pineapple, tea or peanuts, concentrated sulphuric acid (H₂SO₄) or spirit are used.

Precautions

- Before commencing the work, one should wash hands with soap and rinse with absolute alcohol and preferably use sterile gloves.
- As the above compounds are poisonous and mutagenic, they should be handled carefully or hand gloves worn.

- Excess autoclaving can lead to **browning of the media or degeneration of the auxins**.
- Excess of disinfecting (higher concentration and duration) with HgCl_2 or sodium hypochloride will result in **browning of the inoculated plant parts or seeds may lose its viability**.
- Incomplete sterilization or autoclaving of liquid media may **lead to contamination** of media by microbes.
- If pressure cooker is used for autoclaving, it needs standardization of duration or **number of whistles** by the safety valve for getting contamination free cultures.

(b) Aseptic Culture of Microbes

There are three different steps for aseptically transferring of microorganisms:

(i) **Sterilize the Inoculating Loop** The inoculating loop is sterilized by passing it at an angle through the flame of a Spirit lamp or Gas Burner until the entire length of the wire becomes orange from the heat. In this way all contaminations on the wire are removed. Never lay the loop down once it is sterilized.

(ii) **Remove the Inoculum** Removing *inoculum* from a *broth culture* (organisms growing in a liquid medium).

- Hold the culture tube in one hand and in your other hand, hold the sterilized inoculating loop as if it were a pencil.
- Remove the cap or plug the pure culture tube with the little finger of your loop hand. Never lay the cap down or it may become contaminated. (Fig 3.2.1; page 84)
- Flame the rim of the culture tube for half a min. This creates a convection current which forces air out of the tube and prevents airborne contaminants from entering the tube. The heat of the spirit lamp or gas burner also causes the air around your work area to rise and this also reduces the chance of airborne microorganisms contaminating your cultures.

- Keeping the culture tube at an angle, insert the inoculating loop and remove a loopful of inoculum.
- Again flame the rim of the culture tube and replace the cap or plug.
- For removing the inoculum from a **plate culture** sterilize the inoculating loop in the flame and then lift the lid of the culture plate slightly and stab the loop into the agar away from any growth to cool the loop. Scrap off a small amount of the organisms and close the lid.

(iii) Transfer the Inoculum to the Sterile Medium

For transferring the inoculum into a broth tube, pick up the sterile broth tube and remove the cap with the little finger of your loop hand. Do not set the cap or plug down.

- Briefly flame the rim of the broth tube and place the loopful of inoculum into the broth. Withdraw the loop.
- Again flame the rim of the tube and replace the cap.
- Resterilize the loop by placing it in the flame until it is orange and then only lay the loop down until it is needed again.

However, while transferring the inoculum into a petri plate, dry the plate with a sterile swab if it appears to be wet.

- Lift the edge of the lid just enough to insert the loop.
- Streak the loop across the surface of the agar medium. These streaking patterns allow you to obtain single isolated bacterial colonies originating from a single bacterium (Fig. 3.1.1).
- In order to avoid digging into the agar as you streak the loop over the top of the agar you must keep the loop parallel to the surface. Always start streaking at the 12.00 o'clock position of the plate and streak side-to-side as you pull the loop gradually towards you.
- Remove the loop and close the lid. Desterilize the loop before putting it down.

Precautions

- All inoculations may be carried out inside the laminar air flow cabinet.

- After inoculation all broth tube or petri plate should be marked with a Marker pen mentioning—date, source of inoculation, batch number, etc.
- Always keep the broth tube in an upright position. In case the broth culture tubes are solidified with agar-agar, 45° slants should be prepared.

Use of Laminar air flow: Laminar flow, sometimes known as streamline flow, occurs when a fluid flows in parallel layers, with no disruption between the layers. In fluid dynamics, laminar flow is a flow regime characterized by high momentum diffusion, low momentum convection, pressure and velocity independent from time. It is the opposite of turbulent flow. In nonscientific terms laminar flow is “smooth,” while turbulent flow is “rough.” A laminar air flow system contains three basic elements—a blower, a high efficiency particulate air filter (HEPA filters, with high efficiency to remove 99.97% of particles that are up to 0.3 μm in diameter), a UV light provision, and a plenum. There may be variations on this idea—many blowers, many filters, and very large plenums, but all have the same basic features. The flow is called laminar because the turbulent air upstream is changed by the filter into a straight-line flow of the downstream face of the filter.

The HEPA filters are tested by the DOP-diactylphthalate-method when manufactured. DOP, a liquid plasticizer, is heated to the point of vapourization and reconstituted into 0.3 micron particles to form a monodisperse aerosol. These single-size particles are diluted with air until a concentration of 100 mg/l is reached, and the aerosol-air mixtures passed through the filter. The amount of penetration is measured on the downstream side with a forward light scattering photometer, giving the familiar readings of 0.03% or better. The material used to make the filter material is tested in the same way by the filter material manufacturer.

All the infective and microbial cultures and its media, as well as autoclaved plant or animal cell culture media and experimentation, should only

be performed on the platform of the laminar air flow, which provides sterilized non-contaminated air. The UV light should be switched on for at least 15–20 min before and after the use of the laminar air flow, but should be switched off while working on it. The air flow should be kept in a switch on mode at the time of work.

(I) Autoclaving

Autoclaves are also utilized in sterilization. The advantage of using autoclave is that it can reach temperatures higher than boiling water, so it can kill not only bacteria but also bacterial spores, which tend to be resistant. Autoclaves are used in laboratories to assure that items such as culture vessels, glassware, tools, surgical equipment, nutrient media and reagents are sterile.

The maintenance of a sterile environment is very important during the transfer and culture of microbes, plant and animal cells as most nutrient media support growth and get contaminated by unwanted growth of bacteria, and fungal and algal microorganisms, which spoil the culture media. There are several sterilization techniques used for various purposes. Wet heat sterilization is one of the most common techniques applied in the laboratories. Autoclave (which functions like a pressure cooker) is used for this purpose. Autoclaves use pressurized steam to destroy microorganisms, and are the most dependable system available for decontamination. For efficient heat transfer, steam must flush the air out of the autoclave cabinet.

History A rudimentary autoclave was first created in 1879 by the French microbiologist Charles Chamberlain. He worked with the famed Louis Pasteur in his research on sterilization and pasteurization.

Procedure Autoclaves are built around the principle that the boiling point of water increases when it is under pressure. At 15 lbs of pressure per square inch, the boiling point of water increases from 100°C to 121°C. At this temperature, all life forms are killed within 15 minutes.

Autoclaves are filled with water and work by creating steam within an enclosed environment,

which builds up pressure. The air within the autoclave is gradually replaced with steam, which can reach higher temperatures than the air. High-temperature steam can surround and infiltrate the items, even reaching within the crevices in stainless steel instruments.

Autoclaving allows materials to be sterilized within a relatively short time frame without the use of reagents. It also allows objects such as surgical and other equipment to be reused. Therefore, autoclaving is an environment-friendly option. To assure the autoclave has sterilized the objects within it, it is important to note the maximum temperature and how long it was held there. There are also indicators that can be purchased and placed on the items in the autoclaves, which will change colour when they have been held at the appropriate temperature for a certain length of time. Different types of autoclaves are available with different capacity, e.g. horizontal, vertical, portable, etc.

Precautions

- Autoclaves can be extremely dangerous. Pressure builds up within the autoclave and if the door is not sealed properly it can open with great force and cause injury. If liquids are autoclaved, care must be taken to have adequate head space in the container and do not have it sealed. Liquids also must be cooled slowly after the process is finished.
- Before using the autoclave, check the drain screen at the bottom of the chamber and clean if blocked. If the sieve is blocked with debris, a layer of air may form at the bottom of the autoclave, preventing efficient operation.
- Though polypropylene bags are tear resistant, they can be punctured or burst in the autoclave. Place these bags in a rigid container during autoclaving. As it is resistant to heat transfer, it may take a longer time.
- Stainless steel is a good conductor of heat and is less likely to increase sterilizing time.
- Leave space between items to allow the circulation of steam.

- Take into account the size of the articles to be autoclaved. A 2 l flask containing 1 litre of liquid takes longer to sterilize than four 500 ml flasks each containing 250 ml of liquid.
- Autoclave bags containing biological waste should be autoclaved for 50 min to assure decontamination.
- Slowly open the autoclave door. Beware of a rush of steam. After the slow exhaust cycle, open the autoclave door and allow the liquid to cool for 20 min before removing.
- If paper or aluminium foils are used for wrapping test-tubes, petri dishes containing culture media or equipment during autoclaving, the objects should be placed in the oven for a few minutes at 60°C in order to evaporate the condensed moisture.
- Over autoclaving of liquid or culture media can lead to chemical change of liquid and browning of the media.

(m) Distillation

The purpose of this Section is to expose you to the terminologies used in distillation practice and to give a very basic introduction to it.

Distillation is defined as a process in which a liquid or vapour mixture of two or more substances are separated into its component mixture fractions of desired purity, by the application and removal of heat.

Or

Distillation is the process by which two or more liquids in a solution are separated by boiling the liquid with the lower boiling point and condensing it in another flask.

Distillation is based on the fact that the vapour of a boiling mixture will be richer in the components that have lower boiling points. Therefore, when this vapour is cooled and condensed, the condensate will contain more volatile components. At the same time, the original mixture will contain more of the less volatile material. Distillation columns are designed to achieve this separation efficiently. Although many people have a fair idea of what “distillation” means, the important aspects of distillation is from the manufacturing point of view are that:

- Distillation is the most common separation technique.
- It consumes enormous amounts of energy both in terms of cooling and heating requirements.
- It can contribute to more than 50% of plant costs.
- The best way to reduce operating costs of existing units is to improve their efficiency and operation via the process of optimization and control. To achieve this improvement, a thorough understanding of distillation principles and how the distillation system is designed is essential.

The following are the basic principles behind distillation:

- The **bubble-point** is the temperature at which the liquid starts to boil.
- The **dew-point** is the temperature at which the saturated vapour starts to condense.
- This difference between liquid and vapour compositions is the basis for distillation operations.

History

Distillation has been the source of pure water for centuries. Over 2000 years ago, Julius Caesar used solar distillation to produce drinking water for his soldiers. Parts of our world today continue to produce fresh water from sea water, simply by using the sun's heat.

Until the late 1960s, water distillation systems were mainly commercial, used on ships to convert sea water to drinking water and used in laboratories, pharmaceuticals and cosmetic firms. However, as environmental pollution increased, health-conscious people saw a future for residential water distillers.

The Distillation Process Distillation has been an essential part of nature since the earth began. The heat of the sun evaporates water from the earth's surface into the atmosphere, leaving impurities behind. As the vapour cools, it condenses and falls back to earth as rain, snow or other forms of precipitation.

Water distillation is the process of boiling water in a chamber creating steam. As the vapour rises, it passes through cooling coils and collects as pure water. All of the contaminants are left behind in the boiling tank and gases that vapourize at temperatures lower than the boiling point of water are released through volatile gas vents. In essence, distillation duplicate's **Mother Nature's cycle** of evaporation and precipitation and is highly effective in removing all inorganic, organic and radio-nucleotide contaminants. These include heavy metals, ammonia, nitrate, chloride, fluoride, industrial organic contaminants, and pollutants. Distillation is also highly effective in removing commonly used insecticides, herbicides, and lead as well as all bacteria and viruses.

Importance

- The first residential water distillers were designed primarily to remove dissolved solids and inorganic salts. As more 'sophisticated' chemicals were created in our society, the need evolved for 'volatile gas release vents' and 'pre' or 'post' filtration.
- Charcoal filters provide double assurance that 'volatile' contaminants, which may escape being vented, do not end up in the distilled water storage tank. These filters do not breed bacteria, as only pure water passes through. Distillers may also have a self-sterilizing feature for more protection.
- Distillation systems offer their users consistently high quality water and, when used according to the manufacturer's instructions, offer rejection rates exceeding 95% of contaminants. Our systems have a proven rejection rate of over 99%.

Uses of Distilled Water

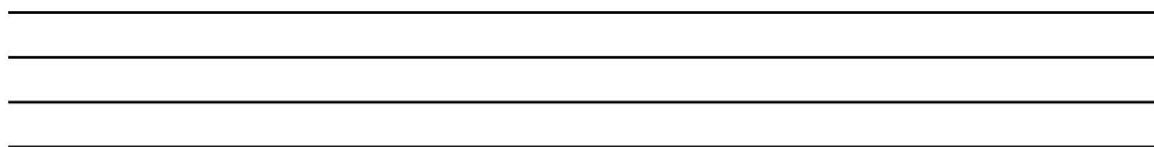
- Provides us with the purest drinking water, free from chemicals, impurities, pollutants and bad tastes.
- Preparation of foods—Clear ice cubes, better tasting tea and coffee, all cooking and baking, better juices and soups, excellent for mixing powdered milk, juice mixes and

dehydrated soups, vegetables taste better, use it for all infant care, excellent for low sodium diets or sensitive stomachs, use it for canning fruits and vegetables.

- Wine-making and mixing drinks

- Spraying house plants (no spotting on leaves)
- Clean contact lenses
- Steam irons, humidifiers, vapourizers
- Car batteries

Notes and Sketches



2

Genetics



Experiment I

Aim Study of various stages of meiosis and mitosis and calculating the Mitotic Index.

Key Terminology

- **Inheritance mechanism** To understand the behaviour of chromosomes during meiotic division to know the protocol of separation and union of alleles from one generation to the next.
- **Sexually reproducing** An offspring arising from a combination of equal amount of genetic material from two parents.
- **Allele(s)** Genes for a particular character situated at a particular locus on a pair of homologous chromosomes.
- **Gamete** Mature sex cell, haploid, the product of a meiotic division, e.g. sperm, ova, pollen nucleus, ovule.
- **Pattern of inheritance** The manner and probability of recombination of alleles which determines the behaviour of offspring in a particular fashion.
- **Gametogenesis** The process which results in the formation of reproduction units (gametes).
- **Reduction division** Change in diploid state to haploid state due to first meiotic division.
- **Meiotic division** A form of cell division occurring only at gamete formation whereby the chromosomes of homologous pairs, and therefore pairs of alleles, are separated into haploid gametes.
- **Mitotic division** Cell division associated with growth; no pairing of homologous chromosomes but chromatids separate into daughter cells which therefore have the same genetic complement as the parent cell.
- **Asexual reproduction** Multiplication of somatic or vegetative cells as a result of mitotic division.
- **Mitotic index (MI)** An index that furnishes a number of mitotic cells undergoing division; it's a ratio between number of different stages of mitotic division by total number of dividing and non-dividing cells.
- **Cytokinesis** Separation of cell contents during division, e.g. cytoplasm, golgi bodies, etc.
- **Cell cycle** The process responsible for continuity of cell growth which indicates the time schedule of a cell type replicating its genetic material, nuclear and cytoplasmic separation taking place during mitotic division. It has been divided into four phases, viz. G_0 , G_1 , S and G_2 .
- **Meristamatic** Somatic shoot or root tips undergoing rapid mitotic cell division.
- **Interphase** Period in between a cell cycle or preparing period when a cell is metabolically most active before undergoing cell cycle. Also known as 'resting phase'.

Basic Principle

Meiosis An organism forms gametes, for example spermatozoa and ovum, in animals, whereas pollen grains and embryo in flowering plants, takes place through meiotic cell division. In meiosis an understanding of the behaviour of chromosomes during division is important to understand the inheritance mechanism. An

offspring is formed by the combination of equal amount of genetic material from two parents and is itself genetically unique. Thus a sexually reproducing population shows greater variation and offers more promising material for rapid evolution by natural selection.

The genetics of higher, diploid ($2n$) organisms will be dealt in this manual. In the diploid or somatic nucleus of a mammalian liver cell for example, or a leaf mesophyll cell, the chromosomes exist in homologous pairs. The member of a homologous pair is identical with respect to length, shape, position of centromere, and gene loci. However, they may differ with respect to the kind of allele, or gene that occupies the locus. As alleles for a particular character (or trait) occur in pair at the same locus of each chromosome, either in homozygous (may be dominant or recessive) or heterozygous state. These alleles, as a result of meiotic division, likewise get separated into different gametes (or haploid state). Thus for any pair of alleles, 50 per cent of the gametes (dominant or recessive allele) will be provided by one parent and the remaining 50 per cent of the gametes (other allele) will be contributed by the other parent. It is the manner and probability of recombination of allelic pairs that form the main exercise of elementary **pattern of inheritance** in all higher organisms.

Thus the whole process of gamete formation (also known as **gametogenesis**) begins from the diploid state, changes to haploid state due to reduction division (or first meiotic division) and ends in a diploid state by forming a zygote as a result of fertilization (Fig. 2.1.1). It is essential to understand the life cycle of a diploid organism to follow the inheritance pattern. Like mitotic chromosomes, meiotic chromosomes may be stained with 1 or 2 per cent aceto-carmin and different stages can be observed under a microscope (for details consult any book on Cytology, Cytogenetics or Genetics for different stages of meiosis).

Mitosis In 1870s, Walther Flemming noted that during cell division, thread-like features were distributed to each of the new daughter cells. He termed this process **mitosis** or the process of the threads (from *mitos*, the Greek word for thread).

Waldeyer named these threads 'chromosomes' in 1888. We now know that the purpose of mitosis is **to distribute genetically identical copies of genetic materials** from parents to each of the two daughter cells. The unicellular organisms undergo mitotic division and increase in number. Mitosis occurs many times in a life cycle and is associated with growth. All vegetative propagated plants are genetically alike and as such have resemblance with each other, e.g. onion bulbs, lily, sugarcane, potato, etc. Such **asexual** reproduction leads to reduction of variability in a population. During mitosis cell division, there is change in the nucleus, which passes through a series of easily recognizable stages. In a resting cell, the chromosome appears as a thin thread, which gradually thickens due to condensation of chromosomes and starts migrating to the equatorial plane. The mitotic chromosomes also show splitting throughout the length (into two **chromatids**) while remaining attached at the centromere point. From the equatorial plane, the chromosome centromere also splits and the sister chromatids start moving towards two opposite poles of the cell, where they rebuild the two daughter nucleus. The cell wall develops between the two nucleus (cytokinesis) resulting in the formation of two daughter cells and completes one cell cycle of a mitotic division (consult any textbook on Cytology, Cytogenetics or Genetics for different stages of mitosis).

In this experiment we will study different stages of mitotic cell division from onion root tips and calculate the **Mitotic Index (MI)**. The process of mitosis is basically the same in all organisms, i.e. plants and animals (only difference is in formation of centrioles in animals and without centrioles in plants).

There are different stains (viz. 1 or 2 per cent Aceto-orcein, Fuelgen, Fluorescent, Gimsa-C and R bands) with which mitotic chromosomes can be stained and clearly observed under a microscope. We will use Aceto-orcein stain which only stains the heterochromatin regions of the chromosome. Whereas, non-heterochromatin regions take light stain and centromere do not take any stain. However, Fuelgen and Gimsa stain DNA, and each of these stains has its importance, application and advantages.

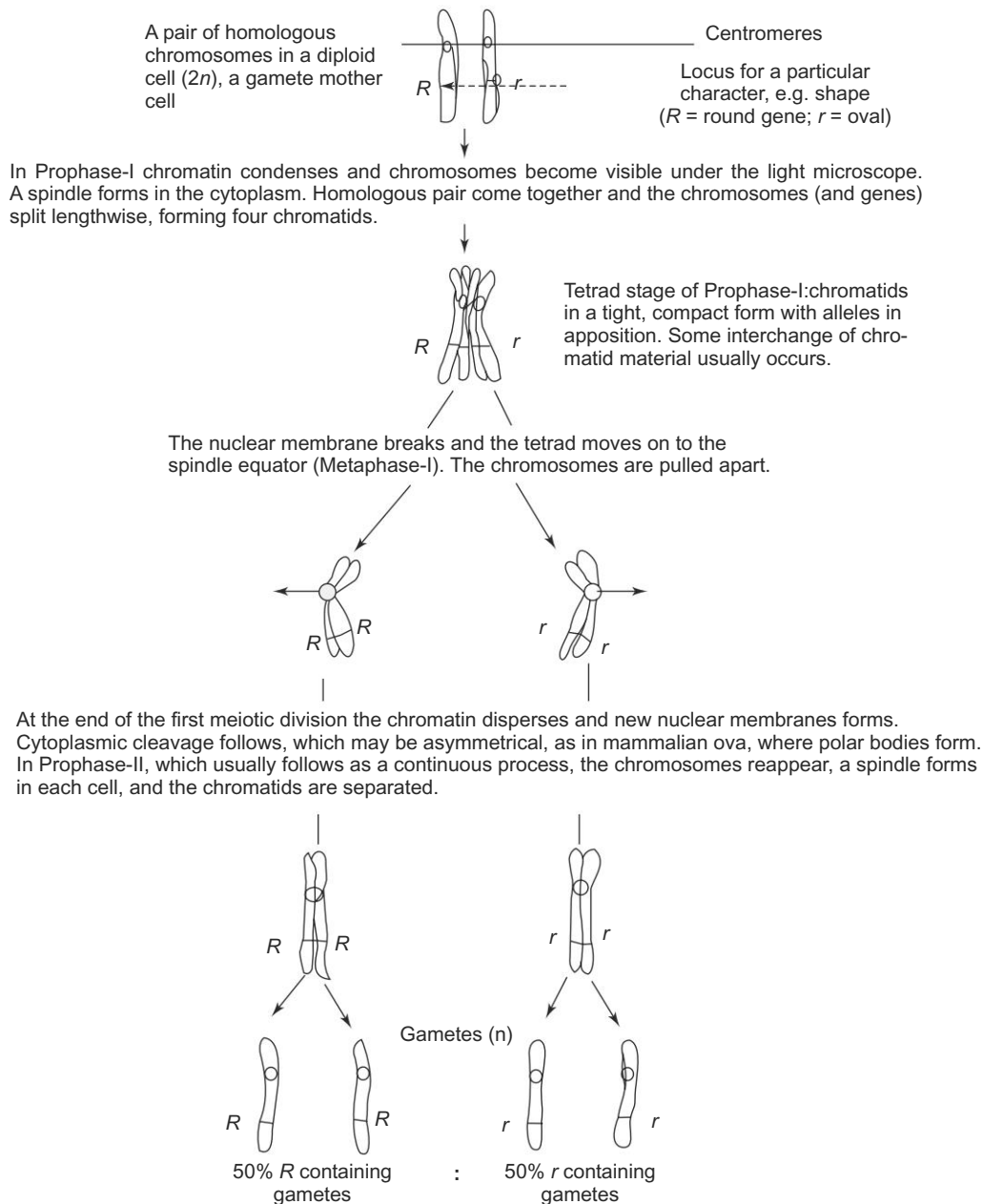


Fig. 2.1.1 A pair of homologous chromosomes showing gametogenesis

Cell Cycle Growing of cells, replicating their genetical material and dividing through a cell cycle process is responsible for continuity of life. Some cells lose the capacity to divide with maturity or divide rarely, e.g. cancer cells. Whereas, other cells

divide rapidly, e.g. meristematic shoot tips or root tips. In a dividing cell population, high proportion of the cells are in **Interphase** (or termed **Resting phase**) and takes 15 to 24 h, whereas, only a few cells are in mitotic phase (or physical change of

chromosomes) which is of a very short duration, usually taking less than an hour. One way to estimate cell division cycle is to calculate the MI. It is ironical to mention Interphase as Resting phase, because the most vital functions of a cell cycle takes place during this period. This phase may be divided as G_0 (G stands for gap), G_1 , S (DNA synthesis) and G_2 . During which metabolic preparations take place.

Materials

- Compound microscope (One microscope for a group of five students)
- Microscope slides
- Cover slips
- Dissecting needles
- Small scissors or sharp scalpel
- Spirit lamp or gas burner or incubator (maintained at 60°C)
- Forceps
- Onion bulbs growing in beakers
- Absolute alcohol and 70% alcohol
- Acetic acid and 45% acetic acid
- 1 or 2 per cent aceto-carmin for meiosis or aceto-orcin (for root tips) in a dropping bottle
- 1N.HCl
- Small test-tubes
- Watch-glass
- Whatman No.1 filter paper

Methods

For Meiosis

1. **Preparation of stain** Take 100 ml of 45% acetic acid, and add 2 g of carmine or orcin powder and boil for 2 min. Cool the content and repeat the process 2 to 3 times. Keep it overnight and filter through Whatman No.1 filter paper. For economy prepare 50 ml of stain.
2. Meiotic cell divisions can be observed in PMCs. Usually at the time of anthesis (generally after sunrise) flower buds are fixed in 1:3 Aceto-alcohol for 24 h. Transfer the fixed PMCs to 70% alcohol and store in the refrigerator. Generally, flower buds of onion (*Allium cepa*), black cumin (*Nigella sativa*) tomato (*Lycopersicum esculentum*) and panicle or spike of barley (*Hordium vulgare*), maize (*Zea mays*), etc. are good materials for observing various stages of meiosis.
3. Select a flower bud and from it with the help of dissecting needles, bring out an anther. Put the anther on a clean slide and crush it. PMCs will come out. Put a drop of 2% aceto-carmin and cover it with a cover slip. Tap it gently with a match stick for dispersal and well distribution of PMCs.
4. Observe the slide under the microscope for dividing PMCs. If it is round pollen grains (indicating anther is mature one) select another from different bud of almost the same size and repeat step 2 and continue till you observe a dividing PMCs. It is a matter of practice and experience to find dividing PMCs and also improve your preparation.
5. If you find dividing PMCs, manipulate the slide for further improvement by warming and applying pressure on the cover slip with your thumb.
6. Identify different stages of meiosis and draw them in your practical record book using 40X objective and label them.

For Mitosis Put onion bulbs for rooting. Fill 2/3 of a beaker with water and place an onion bulb in it, so that lower portion may touch the water. Leave it for 3 or 4 days. It will show development of roots. Alternatively you may grow black cumine, barley seeds for the same purpose. Use germinating paper or put the seeds between two filter papers in a petri dish and soak the filter paper with water. You may select any other plant seeds having less chromosome number and whose mitotic preparations are good.

1. Expose the germinating roots to the sun for half an hour (as it is in growing condition) and collect about (with the help of scissors) 1 cm from the tip in 1:3 aceto-alcohol (i.e. 1 part of glacial acetic acid and 3 parts of ethyle alcohol). Keep them for 24 h and preserve them in 70% alcohol or refrigerate.
2. Transfer the preserved root tips to 45% acetic acid and keep for half an hour and then take

- a test-tube or watch glass, mix 1 part of (one drop) 1 N.HCl and 9 parts (or nine drops) of 2% aceto-orcein in it for maceration of root tips. Transfer the root tips in the mixture and incubate for 3 to 5 min at 60°C in an incubator or warm it using a spirit lamp/ burner (avoid frothing or boiling).
- After 15 to 30 min take the deeply stained tip portion on a microscopic slide and put one drop of 45% acetic-acid. Put a cover slip on it and hold the two ends of the cover slip with the left hand middle and index finger and gently tap over the cover slip with other hand using the back of a match stick.
 - If maceration is complete, the root tip will be soft and spread immediately by tapping. In case maceration is not complete increase the incubation time. For uniform spreading and dispersal of cells and clear vision of the chromosomes, slightly warm the slide over flame and apply pressure over the cover slip with your right thumb. There should not be any overlapping of the cells in the slide. For better clarity and spreading of chromosomes in the cell, root tips may be treated with 0.02% colchicine solution before fixation.
 - Observe the slide under the microscope. First observe using 10X objective and select the right view and then change to high power to 40X. Various stages of mitotic cell division, i.e. prophase, metaphase, anaphase, telophase as well as interphase (or resting) stage will be visible. Show the preparation to your teacher.
 - If your preparation of slide is good, you may temporarily seal it by applying nail-polish uniformly around the cover slip margins and store it in the refrigerator.
 - Store the left out extra macerated root tips in pure 2% aceto-orcein and keep it in the refrigerator for your next class.

Calculate Mitotic Index (MI)

- The same slide prepared for observing mitotic cell division, may be used for calculating MI.
- Find out the meristamatic region on your slide and count how many cells are there in a microscopic field and how many are in interphase, prophase, metaphase, anaphase and telophase. Use 40X objective for classification. Score as many microscopic fields as you can (approx. ~500 cells).
- Enter your data in the table given below and use it for calculating MI. Also pool data from the whole class and compare it with your data. Also find out the most frequent and least frequently dividing stages.

Data Tabulation

	Interphase	Prophase (P)	Metaphase (M)	Anaphase (A)	Telophase (T)	Total
Your data						
Class data						
Total						

Calculation or Results

$$\text{Mitotic Index (MI)} = \frac{(P + M + A + T)}{\text{Total number of cells (Both dividing and non-dividing)}}$$

Figures

1. Using 40X objective draw the different stages of meiosis in your practical record and label them.
2. Draw (using 40X objective) different stages of mitotic dividing cells in your practical record book and label them.

Conclusion

Usually the mitotic index is higher nearer the root tip region than the region away from it. However, it may vary with time of fixation of root tips and

the physical environment, e.g. light, temperature, etc.

Precautions

- Avoid over-heating or maceration of root tips. Over-heating will disintegrate the chromosomes.
- Time of fixing PMCs is very crucial and it varies from plant species to species.
- For good preparation of mitosis and meiosis, sufficient practice is needed.

Viva Questions

- What is the significance of mitosis and meiosis in plant and animal life cycle?
- How does mitotic index help in cell cycle studies?
- What do you mean by interphase or resting stage in cell cycle?
- In plants and animals where do you find mitotic cell division?
- What are the different stages in a cell cycle?
- How many chromosomes will you find in maize ($2n = 20$) root tip metaphase and pair of bivalents in PMC- metaphase?
- In plants and animals where and when do reduction divisions take place?
- In between asexually and seed propagated species, which will show greater variation and why?
- Differentiate G_1 and G_2 phases of cell cycle.

**Experiment 2****Aim**

Preparing Karyotype of human chromosomes from photograph.

Key Terminology

- **Eukaryotes** Organisms having true nuclei.
- **Prokaryotes** Organisms that lack true nuclei.
- **Chromatin** The nucleoprotein material of a eukaryotic chromosome.
- **Genome** The entire genetic complement of a prokaryote or virus or the haploid genetic complement of a eukaryote.
- **Secondary constriction** Other than centromere the gap or absence of euchromatin region in eukaryotic chromosomes but do not develop any kinetochore or heterochromatin which remains attached to the satellite.
- **Euchromatin** Region of eukaryotic chromosomes that are diffused during interphase.
- **Heterochromatin** Chromatin that remains tightly coiled (darkly stained) throughout the cell cycle.
- **Chromomeres** Dark region of chromatin condensation in eukaryotic chromosomes at meiosis, mitosis or endomitosis.
- **Sex-chromosome** Chromosomes carrying genes primarily concerned with determination of sex, usually termed 'X', 'Y' or 'O'.
- **Karyotype** The chromosome complement of a cell represented diagrammatically or photographs arranged group-wise. Also known as 'ideogram'.
- **Mutation** Sudden change in the structure of a gene which changes the nature of its expression in the phenotype, is inheritable.

- **Cryptic** Minute chromosomal abnormality not easily detectable.
- **Autosomes** Chromosomes other than the sex chromosomes.

Basic Principle

The visual description of the complete set of chromosomes of a typical somatic cell of a eukaryotic organism represents the highest level of organization of the genome. Chromosomes are typically arranged in order of decreasing size or grouped together on four major morphological chromosome types depending on the centromere position and consequent relative arm length and ratio, e.g.

- (1) *Metacentric*: Centromere situated at the centre of the chromosome having arm ratio ranging from 1.0 to 0.8 (approx.).
- (2) *Sub-metacentric*: Centromere divides the chromosome into two unequal arms (viz. short and long) with arm ratio ranging below 0.8 to 0.5 or less.
- (3) *Acrocentric*: Centromere is situated almost at the tip, with an arm ratio ranging below 0.5 to 0.1.
- (4) *Telocentric*: Centromere situated at the terminal end of the chromosome.

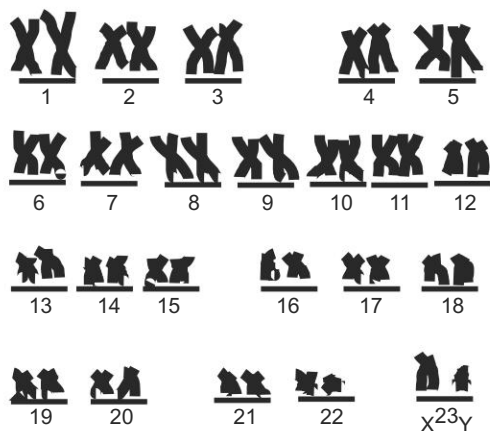


Fig. 2.2.1 Standard chromosomal karyotype of a normal human male (shown in groups)

Beside centromere there are other characteristics of chromosomes, viz. secondary constriction, eu-

chromatin and hetero-chromatin pattern, chromeres, satellited chromosome, and sex chromosomes. Different staining techniques, viz. Fuelgen (also known as DNA stain), fluorescent and gimsa, helps in easy identification of chromosomes. Particularly gimsa stain helps in identifying G, C and R bands in *Drosophilla* salivary and other chromosomes too.

Karyotype analysis may provide important information about an individual or a species:

- Characterization of the evolution of the genome is a powerful approach to solve basic evolutionary questions about natural history, speciation and adaptation.
- Chromosome painting techniques allows the chromosomal position of genes in species.
- Chromosome rearrangements are the building blocks of karyotype evolution that influence genome instability.
- Karyotype evolution provides avenues for the identification of functional constraints operating at mammalian centromeres and therefore, an approach to address how mutation and selection had shaped these essential loci.
- Additions or deletions of whole chromosome sometime occur in karyotyping, indicating abnormality. Some common examples are given below:

Table 2.1 Chromosomal abnormalities in human

ABNORMALITY	NO. OF CHROMOSOMES	AUTOSOME OR SEX CHROMOSOME COMPLEMENT
Normal female	46	XX
Normal male	46	XY
Mongolian	47	Chromosome No. 21 or 22 in triplicate
Klinefetter syndrome	47	XXX
Turner syndrome	45	45 + XO or 46 + XX or 46 + X + del X or 46 + X + rX or 46 + X + X Iso.

- Parental diagnosis of a pregnant mother's (foetus) case of recurrent congenital foetal

anomalies associated with a familial subtelocentric translocation, cryptic chromosome rearrangements can be detected, e.g. chronic myeloid leukemia (CML) results due to translocation between the long arm of chromosome 9 and 22.

- Spectral karyotype (fluorescence) in human dictates chromosome damage in bronchial cells of smokers and patients with cancer.
- Molecular karyotype detection of chromosome abnormality indicates loss of genetic material.

In this experiment we will try to develop a karyotype from the photograph of a human metaphase chromosome. However, karyotype can also be prepared from freshly prepared well spread mitotic metaphase plate of any plants or animals.

Materials

- Photograph of a human metaphase chromosome Plate I and Plate II (given at the end of this experiment (See Plate I and Plate II).
- Scissors and glue.

Methods

1. On the basis of banding pattern and length, chromosome pairs are identified and bands are matched.

Viva Questions



- What different chromosome morphology are used in developing a karyotype?
- How can karyotype studies help in investigation of evolution?
- What is the chromosomal constitution of Klinefelter syndrome and Turner syndrome?
- How do chromosome number and chromosome morphology help in cytogenetical studies?



Experiment 3

Aim

Problems in Mendel's Law of Dominance, Segregation and Independent Assortment.

Key Terminology

- **Inheritance factor** Mendel's inheritable "elements", now known as "gene". The segregation of paired factors and their recombination in a definite proportion in the offspring was described in Mendel's Second Law.

2. With the help of scissors cut out the chromosomes carefully and arrange them in pairs. Now apply glue at the back and paste them on your practical record book.
3. As there are seven groups of autosomes A to G and one pair of sex chromosome (either XX or XY) (See Fig. 2.2.1).

Observations

Karyotype prepared from the photograph of human chromosomes, as it consists of 22 pairs of autosomes and a pair of sex chromosome in Plate I, while Plate II consists of 22 pairs of autosomes and a pair of sex chromosome. Mention male or female.

Result

The karyotype developed from Plates I and II, indicates that it consists of 22 + a pair of sex chromosome.

Precautions

- Match the pair of chromosomes and mark with a pencil before cutting them.
- Arrange the autosomes A to G on the table before pasting them.

- **Recessive** A gene showing no presence, or expressivity, in the phenotype when in the presence of its dominant allele.
- **Dominance** The complete expression of a gene in the phenotype irrespective of its allele; a suppressed allele is termed "recessive". The principle of dominance was

discovered by Mendel and expressed in his First Law.

- **Homozygous** Carrying the same allele at both loci of a homologous chromosome pair, producing only one kind of gamete with respect to that allele.
- **Heterozygous** Having different alleles at any gene locus; a hybrid.
- **Chromatid** The length ways splitting forms a pair of chromatids which ultimately become chromosomes in daughter cells.
- **Monohybrid** Only one pair of allele involved in developing in F_1 or an offspring.
- **Dihybrid** More than one pair of alleles involved in developing an offspring or in F_1 .
- **F_1 or F_2** First offspring generation, F_1 is the first generation, and so on.
- **Phenotypic ratio** Proportion observed based on the appearance of offspring; its physical and chemical features; the manifestation of the genotype (possibly modified by the environment).
- **Genotypic ratio** Proportion observed based on the genetic constitution of an individual with respect to specified alleles.
- **Parental genotype** The appearance of the individual involved in cross; its genetic constitution, with respect to specified alleles.
- **Segregation** The separation of homologous chromosomes, and thereof alleles, into separate gametes or how genes pass from one generation to the next to explain Mendel's first law.
- **Independent assortment** In the formation of gamete the distribution of one pair of alleles does not affect the distribution of other pairs.
- **Test-cross** The cross between F_1 hybrid and a homozygous recessive parent; can be used to test for heterozygous state since a 1:1 ratio is expected.
- **Back-cross** The cross between an F_1 hybrid and with one of its parents repetitively (five to six consecutive generation).

Basic Principles

1. Law of Dominance In his classical experiments with garden peas (*Pisum sativum*) the Austrian monk Gregor Mendel chose an organism in which dominance was complete. He recognized that the hybrid (F_1) plant carried a gene ('**Inheritance factor**') which was suppressed and he termed this **recessive**. The companion gene which was wholly expressed in the phenotype was termed **dominant**.

A gene(s) or trait in a pair of chromosome consists of two forms of alleles (viz. one dominant and the other recessive). During **gametogenesis** a pair of chromosomes splits into four **chromatids**, which gets separated into four gametes. Depending on the parent genotype, a diploid ($2n$) individual may be homozygous (two dominant or two recessive alleles), that will produce gametes which are all dominant or all recessive alleles. However, if it is heterozygous (one dominant and other recessive) it will produce gametes having 50% dominant and 50% recessive alleles. (see Fig. 2.1.1)

Example 2.3.1

In a cross between T (Tall) is dominant and its allele t (Short) is recessive. Thus, since there is no phenotypic distinction between TT and Tt, the $F_1 \times F_1$ (or **monohybrid cross**) yields an offspring phenotypic ratio of 3 tall plants (which consisted of genotype 1TT + 2Tt): 1 short (tt).

Parents: TT (Tall) $\times$ tt (Short)

Gametes: All T All t

↓

F_1 hybrid: Tt (Tall)

$F_1 \times F_1$

(monohybrid cross): Tt (Tall) $\times$ Tt (Tall)

Gametes:

Genotype:

Phenotype :

$\begin{array}{cc} \downarrow & \downarrow \\ T & t \\ \downarrow & \downarrow \\ T & t \end{array}$
 $\begin{array}{cc} \downarrow & \downarrow \\ T & t \\ \downarrow & \downarrow \\ T & t \end{array}$

 $\begin{array}{cccc} T & t & T & t \\ \downarrow & \downarrow & \downarrow & \downarrow \\ TT & Tt & Tt & tt \end{array}$

 3 (1 Tall + 2 Tall) : 1 Short

Problems



- In peas, the gene for tallness (T) is dominant to the gene for shortness (t). What offspring phenotypes would be expected from the following crosses, and in what proportion?
 - heterozygous (Tt) × heterozygous
 - heterozygous × homozygous tall.
- If blue eye colour in man is recessive to other colours, could
 - brown-eyed parents have a blue-eyed child?
 - blue-eyed parents have a brown-eyed child?
- The gene for yellow seed colour coat in peas is dominant to its allele, green. What offspring phenotypic ratio would be expected from a cross between a pea plant known to be heterozygous for coat colour and one which produces green seeds?
- From a mating between two normal-winged *Drosophila* 27 dumpy-winged and 79 normal-winged offspring were produced:
 - What is the nature of the dumpy-winged gene?

(b) What were the parental genotypes?

- Albinism, the inability to synthesise chlorophyll, is a recessive character in a number of plant species. If a tobacco plant known to be heterozygous for albinism is self-pollinated and 600 of its seeds are subsequently germinated,
 - how many seedlings would be expected to be albino?
 - how many would be expected to have the parental genotype?

2. Law of Segregation In parallel with the concept of dominance is the concept of segregation, which can be called **Mendel's Law of segregation**; this states that characters are governed by paired factors (genes) which separate from each other when gametes are formed. Thus a gamete carries only one of any pair of factors. At fertilization the pairs are restored and recombine in definite proportion, i.e. for every 4 (four) individuals (or offspring) formed in F_1 , 3 (three) will have the dominant phenotype and 1 (one) will be recessive.

Solved Problem

The ability to taste phenyl thiourea (phenyl thiocarbamide) appears to be single-gene controlled in man. The taster (dominant) experiences a strikingly bitter taste from an aqueous solution containing 0.005% of the compound which, to a non-taster (recessive) is tasteless even when present in higher concentration.

P_1 :	TT (Homozygous taster)	×	tt (Homozygous non-taster)
Gametes:	All T	↓	All t
	↓		↓
F_1 :	(Heterozygous taster) Tt	×	Tt (Heterozygous taster)
	↓		↓
	T t	↓	T t
F_2 :	TT : Tt : tT	:	tt
Phenotype:	Taster		Non-taster

Problems



- A pair of alleles governs coat colour in the guinea pig; a dominant allele produces black and its recessive allele produces white. Cross between two F_1 black produced 35 black and 11 white.
 - What were the genotypes of the two F_1 individuals?

- b. What is the nature of the black and white alleles?
- c. What further crosses would establish the genotype of these alleles?
7. In *Drosophila* the dominant gene b^+ produces wild type body colour; its recessive allele b produces black body colour. A test-cross of wild-type female gave 105 black and 115 wild-type in the F_1 offspring. If the F_1 female's wild-types are crossed to their F_1 black brothers, what genotypic ratios would be expected in the F_2 ? Draw a diagram of the results using the appropriate genotypic symbols.

In a monohybrid (single gene) cross there may be six different types, e.g. in *Drosophila* sepia coloured eyes are due to recessive allele 's' and wild (red eye colour) dominant allele s^+ .

Cross # 1: homozygous wild having (or red eye colour) $s^+ s^+$ $\times$ homozygous sepia colour ss = All offspring will be wild having $s^+ s$ (or red eye colour) genotype.

Cross # 2: homozygous wild ($s^+ s^+$) $\times$ homozygous wild ($s^+ s^+$) = All offspring will be wild ($s^+ s^+$).

Cross # 3: homozygous sepia colour (ss) $\times$ homozygous sepia colour (ss) = All offspring will be sepia colour (ss).

Cross # 4: heterozygous wild ($s^+ s$) $\times$ homozygous wild ($s^+ s^+$) = All offspring will be wild with 50% $s^+ s^+$ and 50% $s^+ s$ genotype (or 1:1).

Cross # 5: heterozygous wild ($s^+ s$) $\times$ homozygous sepia colour (ss) = 50% offspring will be wild ($s^+ s$) and 50% (ss) genotype (or 1:1).

Cross # 6: heterozygous wild ($s^+ s$) $\times$ heterozygous wild ($s^+ s$) = 25% offspring will be wild ($s^+ s^+$) : 50% offspring wild ($s^+ s$): 25% offspring (ss) sepia colour genotype (or phenotypic ratio will be 3 wild : 1 sepia eye colour).

Out of these crosses, cross # 3 and 4 are also known as a **back-cross** or **test cross**. Any of these cross combinations may be solved or represented using a **branch diagram** or a **forked line**. Another method which can be used is called a **Punnett square** (named after its inventor R. C. Punnett) or

Checker Board.

For a branched diagram see Example 2.3.1:

Punnett square or Checker Board method of representation:

Parents: Tt (Tall) $\times$ Tt (Tall)
 $\swarrow \searrow \quad \downarrow \quad \swarrow \searrow$
 Gametes : $T \ t$ $T \ t$
 F_1 :

	T	t
T	TT	Tt
t	Tt	tt

(iii) **Independent Assortment** Since the alleles are located on different or may be situated on the same chromosome, they behave independently of one another during meiosis. This forms the basis of Mendel's Third Law. The segregation of one pair of gene during the formation of gametes occurs independently of other gene pair. Two or more pairs of alleles show dominance and since the offspring phenotypes for each allele would be expected to show a 3:1 ratio (the monohybrid cross) the offspring ratio of a dihybrid cross is given by: $(3:1) (3:1) = 9:3:3:1$. For instance, the distributions of one pair of alleles Aa , where A is dominant to a , gives an expected offspring phenotypic ratio of $3/4A + 1/4a$. In considering the second pair of alleles Bb , also segregate when both the pairs simultaneously produce offspring by independent segregation of one another.

$(3/4A + 1/4a) (3/4B + 1/4b) = 9/16AB + 3/16Ab + 3/16aB + 1/16ab$ or 9:3:3:1; this may be represented by **Punnett square** or **Checker board**:

	AB	Ab	aB	ab
AB	AABB	AABb	AaBB	AaBb
Ab	AABb	AAbb	AaBb	Aabb
aB	AaBB	AaBb	aaBB	aaBb
ab	AaBb	Aabb	aaBb	aabb

Example 2.3.2

What ratios of phenotypes would be expected in the offspring of a cross between mice heterozygous for both coat colour (where black B is dominant to

brown b) and for wavy coat (where w is recessive to straight W).

Offspring distribution with respect to coat colour = $\frac{3}{4}$ Black + $\frac{1}{4}$ Brown and with respect to hair $\frac{3}{4}$ Straight + $\frac{1}{4}$ Wavy.

Offspring phenotypic ratio when both the traits are segregating = $(\frac{3}{4} \text{ Black} + \frac{1}{4} \text{ Brown}) (\frac{3}{4} \text{ Straight} + \frac{1}{4} \text{ Wavy}) = \frac{9}{16} \text{ Black straight} + \frac{3}{16} \text{ Black wavy} + \frac{3}{16} \text{ Brown straight} + \frac{1}{16} \text{ Brown wavy}$.

Problems



8. In the mouse the gene for coloured coat (C) is dominant to the gene for albino (c) and the gene for straight whiskers (S) is dominant to the allele for bent whiskers (s). Consider a cross between two mice heterozygous for both these alleles, a dihybrid cross, Cc Ss $\times$ Cc Ss:
 - (a) What proportion of the offspring would you expect to be albino?
 - (b) What proportion would have whiskers?
 - (c) What would be the expected ratio of offspring phenotypes with respect to both alleles?
9. If a maize plant heterozygous for the alleles for pigmy and crinkly-leaf (both recessive to normal size of plant and normal leaf) is self-pollinated and 160 of the seeds are subsequently collected and germinated, how many would you expect to show (a) crinkly leaves? (b) normal size leaves? (c) Normal leaves and normal size? (d) Normal leaves and pigmy?
10. If pollen from the maize plant described in the previous question was used to pollinate a plant which showed both pigmy size and crinkly leaves (i.e. was homozygous for the recessive alleles), what offspring phenotypes would be expressed and in what proportion?
11. In *Drosophila*, wild type grey (a⁺) body colour dominant to ebony body colour (a) recessive gene. Vestigial wings (vg) are governed by a recessive gene, and normal wing type (vg⁺) wing size by its dominant allele. A cross between two heterozygous wild type dihybrid flies produced 240 progeny, how many of the progeny flies are expected in each phenotypic class?
12. In garden peas, position of the flowers is governed by a pair of alleles. Flowers growing in the axils (angle between petiole and stem) are due to the action of a dominant allele A, to its recessive allele a, which flowers at the tip of the stem. Second character red flower colour C is dominant over white colour allele c. A heterozygous coloured flower in the leaf axils is crossed to a pure strain of the same phenotype.
 - (a) What will be the genotypic and phenotypic ratios of the F₁ progeny?
 - (b) How will you test the homozygous plants for both the characters?
13. Gene L, dominant for short hair in rabbits and its recessive allele l, responsible for long hair. Brown genotype b recessive to black hair due to the dominant allele B.
 - (a) Find out the expected genotypic and phenotypic ratios in progeny from a cross L/ Bb $\times$ ll Bb?
 - (b) A cross between two rabbits gave 1 Short black : 1 Short brown : 1 Long black : 1 Long brown progeny. Find out the genotypes of the two parents.
14. Find out the different gametes produced by the following individuals:
 - (a) aaBbCc; (b) AaBbCc;
 - (c) AaBbccDd; (d) AABbccDdee.
15. Consider a cross between two pea plants that produced 1277 seeds with the following seed shapes and colours:

715 Round seed, Yellow seed coat;
239 Round seed, Green seed coat;
244 Wrinkled seed, Yellow seed coat, and
79 Wrinkled seed, Green seed coat.

 - (a) What is the allelic relationship between the round wrinkled seeds and yellow vs. green seed coat colour in peas?
 - (b) What further crosses will establish the correct genotype of the two parents?
16. Consider a third character for endosperm, where starchy (S) is dominant over waxy (s),

in addition to those in question 15, and the self-fertilization of a plant heterozygous for all three alleles.

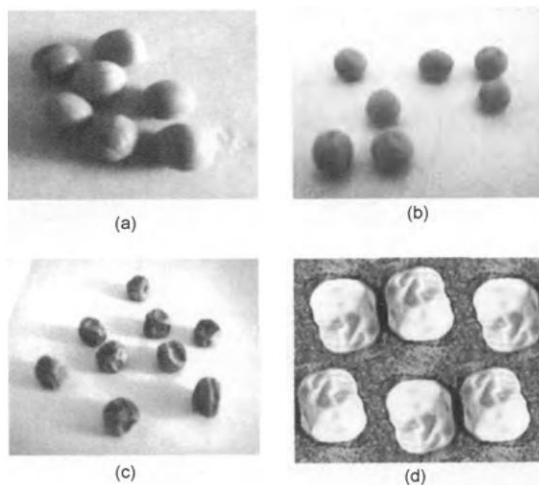


Fig. 2.3.1 Phenotypes showing pea seed shape and colour
(a) green and round (b) yellow and round
(c) green and wrinkled and (d) yellow and wrinkled

Viva Questions



- Can you see the genes situated on chromosomes?
- Between homozygous and heterozygous individuals, which will show segregation?
- How do new rare phenotypes appear in the population?
- What do you mean by pleiotropic gene?
- What is a Test Cross or Back Cross?
- If there are three gene pairs, heterozygous for all three loci, how many different types of gametes will be produced?
- When dihybrids are test crossed, what percentage of their progeny will express same genotype like that of their parents?
- How many genetically different kinds of mating can be made in a population containing two alleles A and a?



Experiment 4

Aim

C-banding technique of *Zea mays* pachytene chromosomes.

Key Terminology

- Euchromatin** It is a lightly packed form of chromatin (DNA, RNA and protein) that is rich in gene concentration, and is often (but not always) under active transcription.
- Heterochromatin** It is a tightly packed form of DNA. Its major characteristic is

- What proportion of the seeds produced would be expected to be round, yellow and starchy?
- What proportion would be expected to be heterozygous for all three alleles?
- What proportion would be expected to be homozygous for all three alleles?

NOTE: In the market, all the four types of dry seeds are available (i.e. round yellow, round green, wrinkled yellow and wrinkled green). These seeds may be mixed in different proportions (genetical ratios) and given to the students for solving the problems.

- In *Drosophila*, curved wing (c) and forked bristle (f) are recessive to the normal or wild type wing and bristle shaped (expressed as + in each case). Show, diagrammatically, the following cases: (a) + c + f × cc ff; (b) + + + f × + c ff; (c) + c + f × + c ff.

- **Chromomere** Is the serially aligned bead or granules like the structure of a eukaryotic chromosome, resulting from coiling of a continuous DNA thread. It is visible on a chromosome during the prophase of meiosis and mitosis.
- **Micro-chromosomes** These are small chromosomes that cannot be easily arranged into a chromogram like the macro-chromosomes. These are telocentric, shorter than normal chromosomes in wheat, corn, etc.

Basic Principle

Chromosome banding has proven to be a valuable tool for karyotype analysis and for rapid detection of gross chromosomal rearrangements, viz.

- large deletions and insertion mutations,
- transcriptions,
- detect amplified regions,
- identify the source of petite micro-chromosomes,
- chromomere** knob pattern, and
- heterochromatin** and **euchromatin** knobs.

In eukaryotic cells, DNA is integrated into complex, condensed chromosomes that contain an intricate matrix of DNA and protein known as chromatin. Chromatin is divided into two classes—heterochromatin which remains condensed throughout the cell cycle and is thought to be transcriptionally inactive, and euchromatin, which is decondensed during interphase to allow gene transcription. When pachytene or metaphase chromosomes are stained, a banding pattern arises that is believed to be due to the stain's differential binding affinity for chromatin structure and DNA sequence.

There are different types of bands, for example:

- C-bands:** which contain tandemly repeated DNA such as centromeric heterochromatin.
- G (Giemsa dark) bands:** most common, which contain late-replicating AT-rich heterochromatin.
- Q-bands** (Quinacrine-binding heterochromatin): which contain some housekeeping genes and tissue-specific regulatory regions, and

- R-bands:** which are early-replicating GC-rich regions that contain most of the known housekeeping genes.

Salivary gland chromosomes of *Drosophila melanogaster* are distinctive cross-banding (appearing when chromosomes are stained) which represent regions (called chromomeres) of the chromatid bundle containing highly coiled or condensed DNA that is interspersed between regions of less condensation. The cross-banding pattern of each chromosome is characteristic and varies in each species.

Simple and less expensive modified C-band technique which gives resolution of the pachytene chromosome knobs of *Zea mays* than acetocarmine, will be explained. The chemical used in this method are not harmful or risky as compared to fluorescent and other banding techniques. With Giemsa the knobs are more distinct in shape and structure and show better resolution. The problems of overlapping chromosomes, chromomeres, etc. can be overcome by this Giemsa method. Giemsa stain does not colour the nucleolus. This method is relatively fast and results in permanent slide. This technique is most useful when dealing with differentiation of corn lines.

Materials

- 2% Gurr improved Giemsa powder
- Farmer's solution
- Absolute alcohol
- Glacial acetic acid
- Barium hydroxide (BaOH_2)
- Gelatin
- Chrome alum
- 0.1 M Phosphate buffer (pH 6.8) (See Annexure II; No. 16a and 16b)
- Distilled water
- Slides and cover slips
- Hot-plate maintained at 37°C (for warming slides and cover slips)
- Camera Lucida
- pH Meter
- Microscope

Methods

The squashing and staining steps are as follows:

1. The *Zea mays* florets are collected and fixed in cold 4°C Farmer's solution of 3 parts of absolute alcohol: 1 part of Glacial acetic acid for 24 h and store in 70% alcohol until preparation.
2. Hydrate the florets in distilled water for 30 min.
3. Remove the anther and place on a subbed slide (subbing agent: 5 g Gelatin, 0.1 g Chrome alum in 100 ml of distilled water).
4. Squash the anthers with a cover-slip in distilled water.
5. Place the slides in a saturated solution of barium hydroxide (BaOH_2) pH 13.5 for 170 min.
6. Remove the slides and rinse 3 times in distilled water.
7. Place them in Giemsa stain (2% Gurr Giemsa improved stain R66 in 0.1 M Phosphate buffer, pH 6.8) for 7 min.
8. Rinse twice in distilled water, air dry and mount cover-slip over permount.

Observations

Observe the prepared slides under the microscope using 10×40 magnifications.

Viva Questions

- How does C-banding Giemsa staining technique help in different chromosome studies of corn?
- Explain the chromosome features of pachytene stage.
- How many bivalent chromosomes will be there in corn (*Zea mays*) pachytene stage?
- What can be interpreted if strains of corn (*Zea mays*) show differences in heterochromatin and chromomeric pattern?

**Experiment 5****Aim**

Problems in Incomplete Dominance Modified Ratios and Multiple Alleles.

Key Terminology

- **Incomplete dominance** The situation in which both alleles of the heterozygote influence the phenotype. The phenotype is usually intermediate between the two homozygous forms.
- **Expressivity (or penetrance)** The degree of expression of a genetically controlled trait.
- **Lethal alleles** Alleles whose summed effect is that of lethality. Whenever the lethal allele is homozygous (recessive allele), i.e. 25% of the offspring do not survive.

Study the pachytene chromosome pattern in each chromosome.

Use different strains of corn lines for comparing heterozygous vs. homozygous knob sequence (or pattern).

It is also possible to develop a karyotype from the Camera Lucida drawing (see Experiment 2).

Results

Overlapped chromosomes, chromomeres, etc. are distinct and clear / not distinct and clear.

Figures

Draw the heterochromatin or chromomeric pattern of different chromosomes with the help of Camera Lucida for drawing karyotype of corn (*Zea mays*). (See Experiments 1 and 2; Pages 31–38).

Precautions

- The time of hydration of florets should be accurate.
- Selection of anther size for squash preparation is very important for pachytene stage. If necessary utilize one anther of a floret in squash preparation with aceto-carmin and check if pachytene stage is present or not. If present use two other anthers of the same floret for squash preparation in distilled water.

- **Genetic interaction (or Modified ratio)** One gene interfering with the action of another gene, resulting in different phenotypic expression or change in genotypic ratio.
- **Epistasis** The making of the action of alleles of one gene by allelic combinations of another gene.
- **Multiple alleles** More than one pair of alleles responsible for expression of a trait.

Basic Principle

(i) **Incomplete Dominance** There are examples where a dominant gene may not completely

suppress an allele, hence a heterozygote is phenotypically distinguishable from either homozygote; both alleles may be expressed in the phenotype. For example, in 4 o'clock plant (*Mirabilis jalapa*), red vs. white flower colour or red vs. yellow flower colour is incompletely dominant, i.e. heterozygotes are pink or orange, respectively. The expressivity (or penetrance) is incomplete. Environmental factors may affect expressivity. There are characters, e.g. coat colour in cattle, testing ability in man, Antirrhinum flower colour, etc.

Example 2.5.1

Inheritance in 4 o'clock plant—

Parents :
Gametes:
F₁ :
F₂ :

RR (Red flower colour) × rr (White flower colour)
All R ↓ All r
Rr (Pink) × Rr (Pink)
↓
1 (RR) Red : 2 (Rr) Pink : 1 (rr) White

Inheritance in Cattle (Cow):

Parents:
Gametes:
F₁ :
F₂ :

RR (Red coat) × rr (White coat)
All R ↓ All r
Rr (Roan) × Rr (Roan)
↓
1 (RR) Red Coat : 2 (Rr) Roan : 1 (rr) White coat

Problems



18. Antirrhinum have pink, white, or red flowers. A number of crosses were made and seeds were collected from each cross. The seeds were germinated and the kind of flowers subsequently produced were recorded as follows:

Crosses		Offspring colour
Red × Pink	gave	126 red and 131 pink
White × Pink	gave	88 white and 92 pink
Red × White	gave	115 pink
Pink × Pink	gave	43 white, 39 red, and 83 pink

What genetic mechanism can account for these results?

19. In radish shape may be long ($S^L S^L$), round ($S^R S^R$) or oval ($S^L S^R$). Long radish plants are crossed to oval radish and the F₁ plants were allowed to cross at random among themselves. What phenotypic ratio is expected in the F₂?
20. When splashed white-feathered chicken are crossed with black-feathered ones, produced state-blue feathered offspring in F₁. When F₁'s are crossed among themselves, it produced splashed white, blue and black offspring in 1 : 2 : 1 ratio, respectively.

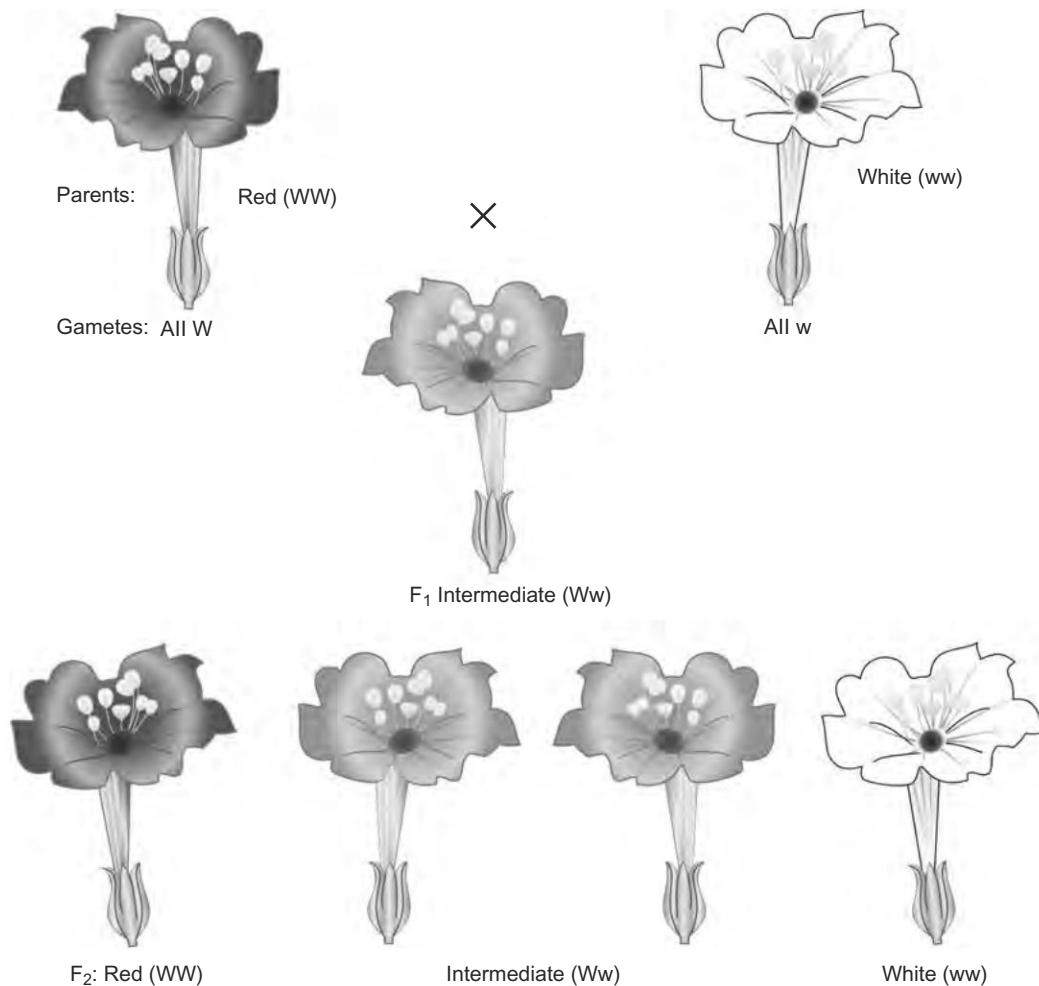


Fig. 2.5.1 Incomplete dominance in 4 o'clock (*Mirabilis jalapa*) plants showing flower colour

- (a) How are feather traits inherited in chickens?
- (b) Use appropriate symbols for these traits and find out the genotype for three different phenotypes.
21. A farmer crossed his roan bull with three groups of cows. Cows of Group A were white; cows of Group B were red; and cows of Group C were roan. What proportions of the different coat colours would be expected in the offspring of each group of cows?

(ii) Lethal Alleles Some genes may cause the death of an organism directly as a result of gross physical or biochemical abnormalities. Others may lower an organism's chance of survival to varying degrees. In some cases lethal genes may be expressed post-embryonically, e.g. albinism (absence of chlorophyll) in tobacco can cause death when the seedling has used up the food store in the seed. Similarly, a gene for kidney malfunction in man causes death after birth when nitrogenous excretion can no longer be carried out by the placenta.

Most lethal genes are recessive. A few, such as those that can be produced by irradiation of *Drosophila*, are dominant. Some show various degrees of expressivity. In vertebrates most lethal of which are expressed as morphological abnormalities appear to be **pleiotropic**, that is, they affect several apparently unrelated parts of the organs, e.g. they may induce bone malformation or mental retardation.

The presence in mammals of a lethal gene which causes death at such an early stage of development that it can be reabsorbed during gestation would yield an observed offspring ratio of 2:1 from a monohybrid cross.

Example 2.5.2

The T locus alleles in the mouse, is in the homozygous state (TT), the embryo shows absence of notochord and mesoderm and dies at about 10 days gestation, being reabsorbed. A cross between mice homozygous for this allele (showing some expressivity as “short-tailed”) would be expected to give an offspring ratio of 2 short-tailed : 1 normal.

Phenotype : Tt (Short-tail) × Tt (Short-tail)

Gametes : $\begin{array}{cc} / & \backslash \\ T & t \end{array}$ $\begin{array}{cc} / & \backslash \\ T & t \end{array}$

Offspring: TT : Tt : tT : tt
1 Dies : 2 Short-tail : 1 Normal

Problems



22. In an investigation of the nature of length of leg in the domestic fowl the following two crosses were made and the numbers of offspring recorded:

	No. of short offspring	No. of normal offspring
(a) Normal × Short leg	48	51
(b) Short leg × Short leg	55	28

From these figures what might be concluded about the action of this gene?

23. In an effort to produce a true-breeding strain of yellow mice a breeder carried out matings

of yellow mice over a number of generations, but always found some offspring of other colours present.

- (a) What might be concluded about the genotype of the yellow mouse at this stage?

On examination of the records of yellow × yellow matings he observed the total number of offspring to be 133 yellow and 68 coloured.

- (b) Construct a diagram for the yellow × yellow mating and offspring.
(c) What is the fate of the homozygous yellow?
(d) How might the breeder verify this hypothesis?

24. In humans thalassemia is a heredity disease of blood anemia. In homozygotes ($T^M T^M$) causes severe anemia (thalassemia major) and ($T^M T^N$) heterozygotes a milder form of anemia (thalassemia minor). $T^N T^N$ homozygous are normal individuals. All individuals with severe anemia die before sexual maturity.

- (a) What proportion of normal will be observed from marriages of thalassemia minor?
(b) What would be expected anemic from marriage of F_1 adults?

25. Seeds from self-pollination of a tobacco plant heterozygous for the recessive allele albino were collected.

- (a) What is the expected green: white seedling ratio?
(b) Do you consider the observed ratio of green: white seedlings adequately confirm to the expected ratio?

(iii) Modified Ratios (or Genetic Interaction)

The phenotypic ratios are modified due to interactions of gene(s). The given environment may affect many gene expressions. It is not only the external environmental factor which is responsible (e.g. temperature, quality of light, etc.) for modification of phenotype but also internal factors, e.g. hormones and enzymes considerably

50 Analytical Techniques in Biotechnology

When two dihybrid F_1 plants were inter-pollinated, 6% of the progeny produced ovoid-shaped seed capsule, whereas,

remaining 94% of progeny showed triangular-shaped seed-capsule.

What two-factor epistatic ratio is approximated by the progeny?

F_1 Parents : $AaBb$ (Triangular shaped) $\times$ $AaBb$ (Triangular shaped)
 $\downarrow$
 F_2 : $\frac{9}{16} A^-B^- + \frac{3}{16} A^-bb + \frac{3}{16} aaB^- + \frac{1}{16} aabb$
 Phenotypic ratio: 15 triangular shaped 1 Ovoid shaped

3. *Duplicate recessive genes*: Absence of both the dominant alleles and recessive ($aabb$) alleles produce same phenotype, whereas, dominant alleles together complements each other resulting in **9:7** ratio.
5. In sweet pea (*Lathyrus odoratus*) two white flowered strains when crossed produced purple flowers in F_1 . Random crossing among F_1 produced 106 purple and 86 white

flowers. What type of interaction is involved in sweet pea?

Conclusion

Total offspring = $106 + 86 = 192/16 = 12$. Therefore, $12 \times 9 = 108$ and $12 \times 7 = 84$. So, it is expected to be a case of duplicate recessive genes which produce 9:7 ratios.

Parents : $AAbb$ (White) $\times$ $aaBB$ (White)
 $\downarrow$
 F_1 : $AaBb$ (Purple) $\times$ $AaBb$ (Purple)
 $\downarrow$
 F_2 : $\frac{9}{16} A-B- : \frac{3}{16} aaB- + \frac{3}{16} A-bb + \frac{1}{16} aabb$
 9 Purple: 7 White

(iii) *Dominant and Recessive Interaction* Out of two interacting genes, only one dominant allele produces change in phenotypic effect (i.e. $A-B-$, $A-bb$ and $aabb$ produce one phenotype while $aaB-$ produce another).

Solved Problem

6. In *Drosophila*, S gene produces an eye colour condition known as 'star'. Their recessive allele S^+ is responsible for wild type eye. The dominant allelic of another locus, i.e. $Su-S$ is capable of suppressing the expression of S . However, recessive allele of $Su-S$ do not have any effect on S^+ .

- (a) Find out the type of interaction.
- (b) What will be the F_1 phenotypic ratio, if a normal eyed male of genotype $Su-S/Su-S$, S/S is crossed to a homozygous wild type female, having $Su-S^+/Su-S^+$, S/S genotype?

Answers

- (a) Dominant and recessive alleles interact to change the eye phenotype; it may be a case of dominant and recessive interaction.

- (b)

Parent : $Su-S/Su-S$, $S/S \times Su-S^+/Su-S^+$, S^+/S^+
 Wild-type male $\downarrow$ Wild-type female

F_1 : $Su-S/Su-S^+$, S/S^+

All offspring will be wild type which is due to presence of $Su-S$ dominant allele

(iv) *Multiple Alleles* In the data considered so far in this chapter, a particular locus on a chromosome has been thought of being occupied by one of two genes (one of an allelic part). A gene locus of any individual possesses maximum two numbers of alleles, with one on each of the homologous chromosomes. There are several examples where

a large number of alleles exist in a population of individuals. If in a population we find a locus having more than two alleles at a gene locus, it is known to be a **multiple allelic series**. However, dominance relationship may be determined by cross-involving multiple alleles. Alternative forms of alleles are formed by the process of mutation. A large number of alleles are theoretically possible in a population, e.g. blood group in human, *Drosophila* eye colour, self-incompatibility in mustard (*Brassica* sp.), coat colour in rabbits etc.

- (a) In ABO blood group in human there are three alleles— I^A, I^B and I , e.g.
 $I^A I^A$ or $I^A i$ = A blood group; $I^B I^B$ or $I^B i$ = B blood group; $I^A I^B$ = AB blood group and ii = O blood group.
- (b) *Drosophila* eye colour— $w^+ > w^{co}$ (coral) $> w^{bl}$ (blood) $> w^e$ (eosin) $> w^{ch}$ (cherry) $> w^a$ (apricot) $> w^h$ (honey) $> w^{bf}$ (buff) $> w^t$ (tinged) $> w^p$ (pearl) $> w^i$ (ivory) $> w$ (white).
- (c) Self-incompatibility— $S_1, S_2, S_3, S_4 \dots$
- (d) Rabbit coat colour— $C > C^{ch} > C^h > c$.
- (e) Chinese primrose—A (Alexandria type = white) $> a^n$ (normal type = yellow eye) $> a$ (primrose Queen type = large yellow eye).
- (f) Plumage colour in mallard ducks— M^R (Restricted mallard pattern) $> M$ (mallard) $> m$ (Dusky mallard).
- (g) Coat colour in cattle—(Band of white colour in the middle of the animal or Dutch belt) $> S^h$ (Hereford spotting) $> S^c$ (Solid colour) $> s$ (Holstein-type spotting).

Problems



26. In a certain marriage the first child was born normally, the second suffered from severe

haemolytic disease, the third was stillborn, and the fourth was normal.

- a) On the assumption that the rhesus factor was responsible for these misfortunes give the possible genotypes of the parents and children.
 - b) If a further child was conceived, what is the probability that it would suffer from haemolytic disease or be stillborn?
27. In the rabbit, chinchilla coat (c^{ch}) is dominant to Himalayan coat (c^h). The albino gene (c), however, is recessive to both of these alleles. All these genes are recessive to C , the gene for full coat colour. What offspring phenotype ratios would be expected from the following crosses?
 (a) $c^h c^{ch} \times c^h c^{ch}$,
 (b) $c^{ch} c \times c^h c$ and
 (c) $Cc \times c^h c^{ch}$.
 28. A rabbit breeder wishes to buy a fine chinchilla buck from a colleague but he suspects that it may not be homozygous at the C locus.
 (a) How could he arrange for this uncertainty to be eliminated?
 (b) If he mates the buck with a pure-breeding Himalayan doe and obtains a litter of 6, all chinchilla, how sure would he be that the buck is homozygous for the chinchilla gene?
 29. A *Nicotiana* plant carries self-sterility alleles $S_2 S_4$. If pollen from flowers of the following list of plants is transferred to the stigma of this plant, what proportion (in percentage) is likely to germinate a successful pollen tube and what offspring genotypes will be found in each case?
 (a) $S_1 S_2$; (b) $S_2 S_3$; (c) $S_1 S_3$; (d) $S_2 S_4$ and
 (e) $S_3 S_4$.

Viva Questions



- What is meant by dominant and recessive interaction?
- What is the difference between epistatic and hypostatic gene?
- The ABO blood groups of human are determined by three alleles. How many genotypes are possible for these phenotypes?
- A mother of blood group O has a group O child. What is the blood group and genotype of the father?
- What is self-incompatibility?

- In a cross between $S_2S_4 \times S_1S_4$, what percentage of different genotypes will appear?
- "Homozygous results in the death of the individual during development", what is this gene known as?
- What is a gene which is not completely dominant and produces an intermediate phenotype is known as?



Experiment 6

Aim Problems in Linkage.

Key Terminology

- **Linkage** Linkage is a phenomenon which results in movement of linked genes in a chromosome to move together in the formation of gametes resulting in more frequent appearance of parental combination than the recombinant type.
- **Recombination** The non-parental arrangements of alleles in progeny that can result from either independent assortment or crossing over.
- **Coupling phase** Allelic arrangements in which mutants (recessive) are on the same chromosome and wild-type (dominant) are on the homologue.
- **Repulsion phase** Allelic arrangements in which each homologous chromosome has mutant (recessive) and wild-type (dominant) alleles.
- **Map distance** A map distance represents one per cent recombination; the units reflect the distance apart of linked genes on a chromosome.
- **Cross-over value** The percentage recombination of linked genes; one per cent represents one map unit on the chromosome map.
- **Genetic mapping** The process of locating the position of genes on chromosome based on calculated map unit distances.
- **Interference** The presence of a chiasma in a meiotic prophase-I tetrad inhibits or interferes with crossing-over for some distance each side.

- **Coefficient of coincidence** The number of observed double. Cross-overs divided by the expected based on the independent occurrence of cross-overs.

Basic Principle

Mendel's Law of Independent Assortment refers only to non-linked genes, i.e. alleles carried on different pair of chromosomes. When genes lie on the same chromosome, however, they tend to pass into the same gamete and are said to be linked. In such circumstances an F_1 dihybrid cross would not give a 9:3:3:1 ratio. Therefore, linkage is a phenomenon which tends the movement of the genes together during meiosis (or without any crossing over) resulting in higher frequency of parental types than the recombinant types. Whether the gene situated on the same chromosome is considered to be linked or showing Mendelian segregation (i.e. Independent Assortment) depends on the spatial distance between loci. Closer the genes are situated in the chromosome, higher will be their tendency to move together in a gamete (or to say proportional to the distance between the genes). Interchange of chromatid material within a tetrad is known to take place during prophase-I of meiosis. At a crossover point (chiasma or point of genetic exchange), where breakage between linked genes occur, they get separated and reconstitution of chromatids takes place, which is termed as **recombination**.

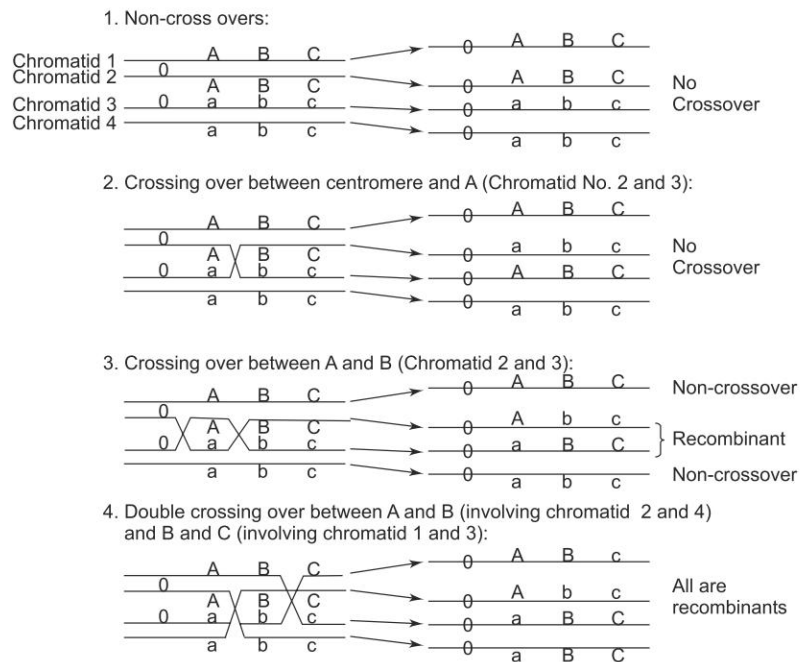
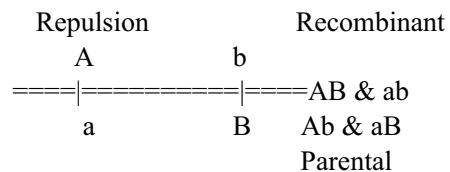
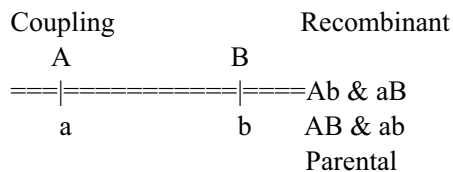


Fig. 2.6.1 Recombination involving different chromatids

Linked genes do not assort independently. They always have a tendency to move together, as they are situated in the parents. In a test cross dihybrid, we expect 1:1:1:1 ratio in the progeny, however, linked genes do not show any such evidence. There are two types of linkage relationship. It is decided by the situation of dominant and recessive alleles

on the same chromosome. If the two dominant (or wild type) alleles are on one chromosome and recessive (or mutant) alleles on the other, it is termed as **coupling phase**, whereas one dominant and one recessive are situated on the chromosome, it is known as **repulsion phase**.



All genes situated in the chromosome forms a linkage group, e.g. the diploid number of chromosomes in man is 46, therefore, there are 23 linkage groups.

Problems



30. An animal heterozygous for three alleles (genotype Aa, Bb, Cc) lays 400 unfertilized

eggs of which 48 are abc, 51 are aBC, and 50 are Abc. Are these alleles linked or independent?

31. An animal heterozygous for three alleles (Cc, Dd, Ee) is mated with one homozygous for all three recessives (cc, dd, ee) and produces 400 offspring of which 146 are C- D- E-. Is this evidence of linkage?

32. In maize there are 10 pairs of homozygous chromosomes. The recessive gene for *brown-midrib* (*bm*) is located on chromosome V.
- How many linkage groups are there in maize?
 - What is the probability that a second gene, *brevis* (*bv*) (short internodes, making a plant of half-size), will be linked with *brown-midrib*?
 - What would be the offspring phenotypic ratio resulting from self-pollination of a plant heterozygous for these alleles (*+bm +bv*) if the alleles lie on different chromosomes?
 - What would be the offspring phenotypic ratios of that dihybrid cross if *bm* and *bv* were linked with no crossing over?
33. What gametic ratios are expected in an individual heterozygous for a pair of factors in coupling with recombination value 10, 25, 22.8 and 50%?

What will be the gametic ratio in repulsion?

Limits of Cross-over Value or Map Distance

As long as free crossing over takes place between the genes situated in the chromosome, showing Mendelian ratios or have a cross-over value of 50% and above, it cannot be considered the genes to be linked or produce progeny in a 1:1:1:1 ratio.

Generally, the longer the chromosome and genes situated apart, the greater the number of chiasma. However, it is necessary that each chiasma is followed by crossing-over. It is the characteristic of each chromosome within a species showing number of chiasmata. The percentage of cross-over (or recombinant) gametes formed by a given genotype is a direct representation of the frequency with which a chiasma forms between two linked genes. **Cross Over Values** (COVs) give an idea about the position of gene location on the chromosome. Closer the genes, smaller will be the recombination value.

The number of offspring resulting from recombination expressed as a percentage of the total offspring is termed **cross over value** (COV).

$$\text{COV} = \frac{\text{Frequency of recombinant offspring}}{\text{Total no. of offspring}} \times 100$$

The spatial distance between two linked genes can be calculated by recombination percentage or map unit distance, which is often referred to as **centimorgan** (cM) in honour of the great *Drosophila* geneticist T. H. Morgan.

Example 2.6.1

- Genotype $\frac{A}{a} \frac{b}{B}$ produces 105 each of the cross-over gametes AB and ab, which means the distance between A and B is estimated to be 20 map units.
- Map distance between the loci B and C is 8 units, then 8% of the gametes of genotype $\frac{B}{b} \frac{C}{c}$, will be 4% BC and 4% bc.

Example 2.6.2

In maize, cross was made between heterozygous (Cc Ss) coloured, full and homozygous for these alleles (cc ss). 8368 seeds were obtained. When these seeds were grown it showed the following phenotypes:

Coloured, Full (CS/cs) = 4032

Coloured, Shrunken (Cs/cs) = 149

Colourless, Full (cS/cs) = 152

Colourless, Shrunken (cs/cs) = 4035

Since the number of recombinant plant is (149 + 152) = 301; out of 8368.

Therefore, COV = $301 \times 100/8368 = 3.6$

Example 2.6.3

A cross between a female *Drosophila* heterozygous for tarsi-fused (*ti*) and light-eye, and a male homozygous for these recessive genes gave the following offspring:

1. ++	270	2. +lt	2
3. ti +	3	4. ti lt	<u>275</u>
		Total:	550

Recombinants = $(5 \times 100)/550 = 0.9\%$

Example 2.6.4

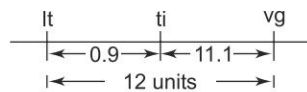
A second cross between a female heterozygous for tarsi-fused and vestigial wing and a homozygous male produced the following offspring:

1. ++	146	2. ti +	18
3. + vg	18	4. ti vg	<u>142</u>
Total: 324			

$$\text{Recombinants} = (36 \times 100)/324 = 11.1\%$$

Therefore by using these results and those from Example 2.6.3 it is possible to arrange these loci linearly:

COV lt/vg = 12%; lt/ti = 0.9%; ti/vg = 11.1%



It is advantageous to cross with a homozygous recessive individual, sowing 1:1:1:1 ratio, which is indistinguishable from independent assortment.

Problems

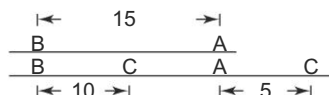
34. A maize plant known to be heterozygous for the alleles (+bm +bv) was fertilized with pollen from a brevis plant with brown midribs; 200 seeds were collected and germinated with the following results:
- | | | | |
|---------|----|----------|----|
| 1. + + | 93 | 2. bm + | 7 |
| 3. + bv | 5 | 4. bm bv | 95 |
- What linkage patterns are present?
 - Show, diagrammatically, the chromosomes in the cross.
 - What is the crossover value bm/bv ?
 - What would have been the answer to (b) had the results been as follows?
- | | | | |
|---------|----|----------|----|
| 1. + + | 6 | 2. bm + | 89 |
| 3. + bv | 99 | 4. bm bv | 6 |
35. A female *Drosophila* heterozygous for the recessive genes blister-wing (by) and spineless-bristles (ss) was mated with a male showing both characteristics in the phenotype. There were four groups of the offspring:
- | | | | |
|---------|-----|----------|-----|
| 1. + + | 222 | 2. + ss | 23 |
| 3. by + | 27 | 4. by ss | 228 |
- What linkage groups are present?
 - What is the COV by/ss?
 - How many offspring showing both blister-wing and spineless-bristles would be expected among the 200 offspring of a mating of a female of group 3 and her male parent?
 - Make a chromosome diagram of this cross.
36. A geneticist, taking account of the results in the previous question, crossed a female fly heterozygous for blister-wing and for another recessive gene, stripe-thorax (sr), with a male homozygous for these alleles. The results were:
- | | | | |
|---------|-----|----------|-----|
| 1. + + | 175 | 2. by + | 27 |
| 3. + sr | 26 | 4. by sr | 172 |
- What is the COV by/sr?
 - Why does not this result (and that related to question 35) enable the three alleles by, ss, and sr to be mapped?
 - To map these alleles what further cross should be made?
37. The same geneticist then crossed a female fly heterozygous for stripe-thorax and spineless bristles with the double recessive male:
- | | | | |
|---------|----|----------|----|
| 1. + + | 97 | 2. sr + | 4 |
| 3. + ss | 3 | 4. sr ss | 96 |
- Map the three alleles ss, sr, and by.
 - How could this geneticist have designed this mapping experiment in an easier way?
38. In a three-point cross between a *Drosophila* female heterozygous for black-body (b), purple-eye (pr), and engrailed (en) and a male homozygous for these three recessive genes, the results were:
- | | | | | | | | |
|---|----|----|-----|---|----|----|----|
| + | + | + | 431 | + | + | en | 37 |
| b | pr | en | 437 | b | pr | + | 35 |
| + | pr | en | 28 | + | pr | + | 1 |
| b | + | + | 29 | b | + | en | 2 |
- What is the COV b/pr?
 - What is the COV pr/en?
 - Draw a chromosome map of these loci given that black-body has the lowest map unit number at 48.5.

(ii) Genetic Mapping Since genes are arranged linearly and in fixed sequence the frequency with which they are separated by crossing over indicates how far apart they are from one another. Map distance derived directly from percentage recombination, helps us to place genes in their proper linear order. In a three-point linkage, e.g. ABC, there may be three different orders, depending upon which gene is situated in the middle.

Example 2.6.5

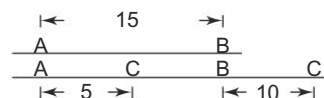
In a three-point linkage, where double cross-over do not occur, map distance may be treated only as additive units. Given the distance between A/B = 15, B/C = 10, and A/C = 5. Find out the correct gene order.

1st possibility: Suppose A is in the middle.



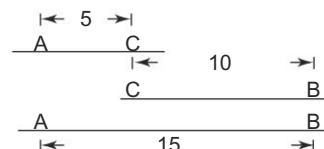
Gene C does not align.

2nd possibility: Suppose B is in the middle.



Gene C does not align.

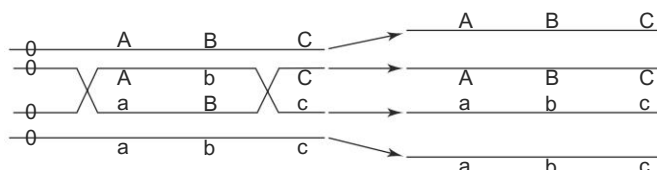
3rd possibility: Suppose C is in the middle.



As all three genes align, therefore, C is in the middle.

Example 2.6.6

The % crossover value should, strictly, be termed recombinant value. For loci close together, such as light-eye and tarsi-fused in Example 2.6.4 above, they are the same, but for loci that are further apart the incidence of crossing over may be greater than the observed recombinant types owing to double crossovers:



Problems



39. Sex linked genes of *Drosophila* echinus (ec) females crossed to scute (sc), cross-veinless (cv) males produced all wild-type females and all echinus male in the F_1 . When F_1 females were test-crossed, produced following offspring (include both male and female progeny).

Echinus	= 830
scute	= 109
scute, cross-veinless	= 848

echinus, scute	= 82
cross-veinless	= 108
echinus, cross-veinless	= 123

Find out the genetic map.

40. The F_1 progeny from a cross between P_1 (cc ShSh wxwx) $\times$ P_2 (CC shsh WxWx) was test-crossed to a colour-less, shrunk, waxy strain. The progeny exhibited the following phenotype:

226	colour-less, shrunk, starchy;
8	coloured, plumpy, starchy;
5416	colour-less, plumpy, waxy;

1252	colour-less, plumpy, starchy;	C.O. between app and cu-	+	cu	sr	58
4	colour-less, shrunken, waxy;			app	+	49
232	coloured, plumpy, waxy;	C.O. between cu and sr-	+	+	sr	55
5076	coloured, shrunken, starchy;			app	cu	48
1202	coloured, shrunken, waxy.	Double crossovers-	+	cu	+	3
Draw a genetic map for these three regions of the chromosome.				app	+	sr
						<u>2</u>
						900
						offspring

(iii) Interference; Coefficient of Coincidence

The presence of a chiasma at a particular locus in a Prophase-I tetrad appear to inhibit other chiasmata for a distance either side (in *Drosophila* it is 10 map units). This interference diminishes at greater distances, depending on the chromosome involved and at what site (e.g. interference is observed close to centromeres). Interference distances also vary from one species of organism to another. The coincidence of two observed cross-overs expressed as a ratio of those expected is termed the **coefficient of coincidence**.

Coefficient of coincidence =

$$\frac{\text{Observed frequency of double cross-over}}{\text{Expected frequency of double cross-over}}$$

Example 2.6.7

The following offspring phenotypes were obtained from a cross between *Drosophila* heterozygous for three alleles, approximated-vein, curled-wing, and striped-thorax (+app, +cu, +sr) and males homozygous for these alleles.

Parental types-	+	+	+	337
	app	cu	sr	348

Crossovers between app and cu (including doubles) = $112/900 = 12.5\%$ or 12.5 units

Crossovers between cu and sr (including doubles) = $108/900 = 12.0\%$ or 12.0 units

Thus, app and cu are 12.5 map units apart and cu and sr 12.0 units apart. If only app and sr were considered the COV would have been $210/900 = 23.3\%$ or 23.3 units apart, instead of 24.5 units.

Since the distance part of a pair of loci is a measure of the probability that crossing-over will occur between them, the probability of a double cross-over (i.e. the expected frequency) is given by the product of the probabilities for each region. Thus, from the last example,

COV app/cu (excluding doubles) = $(107 \times 100) / 900 = 11.9\%$

cu/sr (excluding doubles) = $(103 \times 100) / 900 = 11.5\%$

Therefore expected doubles = $0.119 \times 0.115 = 0.0137$.

But observed doubles = $5/900 = 0.0055$.

Therefore, the coefficient of coincidence = $0.0055/0.0137 = 0.4$.

A coefficient of coincidence of 0 indicates complete interference and 1 indicates no interference.

Viva Questions



- What is the crossed shape cytological structure between homologous meiotic chromosomes called that generally appear in Prophase I?
- Do single crossovers influence double crossovers?
- What are coupling and repulsion phases of genes?
- What is a centigram?
- What will be the value of interference when two gene pairs on the same chromosome is showing Mendelian segregation?
- Do you expect crossing over when the coefficient of coincidence value is 0?

- If in maize $2n = 20$, how many linkage groups will be their?
- How can you determine whether two gene pairs are linked or not?
- What are the uses of linkage maps?



Experiment 7

Aim Problems in Sex-determination.

Key Terminology

- **X or Y chromosome** Chromosomes responsible for deciding the sex-expression (See Experiment 2)
- **Sex-linked gene** Gene carried on sex chromosomes; usually refers to genes on the non-homologous portion of the x-chromosome.
- **Holandric** Trait controlled by a locus found only on the x-chromosome. Involves father-to-son transmission.
- **Carrier** An organism heterozygous for a recessive gene completely suppressed in the phenotype; 50% of the organism's gametes would contain this gene.
- **Non-disjunction** Failure of homologous chromosomes to separate at meiotic Anaphase-I; one daughter gamete receiving two chromosomes and the other none.
- **Secondary sexual characters** Sterile XO female with poor ovary development, webbing of the neck and mental retardation (Turner's syndrome) or XXY sterile male having undeveloped testes and little body hair, some mammary development (Klinefelter's syndrome) are known as secondary sexual characters.
- **Sex-influenced genes** Genes that appear more often in one sex than in another.
- **Sex-limited genes** Genes expressed in only one sex. They may be controlled by sex-linked or autosomal loci.

Basic Principle

In the first decade of the nineteenth century much attention was being focused on cytological microscopy. Particularly, the behaviour of chromosomes during cell division and their role in inheritance was investigated. In 1902, McClung noted the presence of an odd chromosome in the diploid cells of the male grasshopper; this was termed as the **X** (for unknown) **chromosome**. Later it was found that the female insect had a pair of **X-chromosomes**. This discovery led to the investigation for determination of sex-chromosomes in other species. In the males of many animals it was found that the **X-chromosome** paired with a small, non-homologous chromosome at the beginning of meiosis. This was called the **Y-chromosome**.

(i) **Sex-chromosomes in Mammals** In mammals the female sex is homologous for sex chromosomes, XX and the male is heterozygous, XY. In man the chromosomal complement is **one pair of sex-chromosomes plus 22 pairs of autosomes**. The genes located on the non-homologous portion of the X-chromosome, is known as "**sex-linked gene**".

Problems



41. If it become possible to separate wholly, or in part, bull sperm into X- and Y-carrying sperm, this would have great economic value to dairy and beef farmers. What proportion

- of female offspring would be expected from a herd of cow artificially inseminated with a batch of sperm from which half the Y sperm had been removed?
42. If a mammal carries a sex-linked recessive lethal gene causing abortion of the embryo at an early stage when it is expressed, what proportion of its offspring would be expected to be males?
 - (a) What proportion of its female offspring would be carriers?
 - (b) Could an embryo aborted from the action of this gene be a female?
 43. A sex-linked, dominant gene, barred (feather pattern), can be used to sex newly hatched chicks, the offspring of a barred hen mated with a non-barred rooster.
 - (a) What is the phenotype of the female chicks?
 - (b) Construct a diagram to show how this cross operates.
 44. In *Drosophila* Bar-eye (B) is a dominant, sex-linked gene. Show diagrammatically, the cross between a bar-eyed male and a wild-type female, the expected genotypes, phenotypes, and sex of their offspring.

(ii) **Sex Determination in *Drosophila*** In *Drosophila*, sex is determined by the ratio between X chromosomes and autosomes. Unlike the male mammal, the Y chromosome in *Drosophila* seems to carry no male-promoting genes.

- (a) $XX + 3$ pairs of autosomes = Normal female
- (b) XY or $X^- + 3$ pairs of autosomes = Normal male

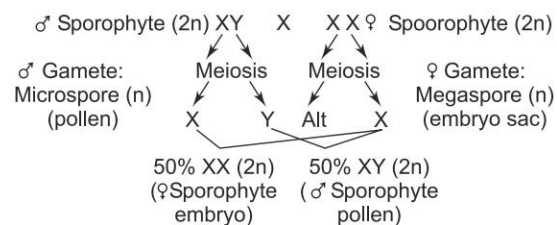
(iii) **Sex Determination in Birds** In birds the male is homogametic sex, having XX sex chromosomes but the female has only one sex chromosome, i.e. X- or XO.

	(Cock) XX	×	XO (Hen)
Gametes:	All X		X O
F ₁ :	50% XX (Males) : 50% XO (Females)		

Fig. 2.7.1 Inheritance of sex-chromosomes in birds

(iv) **Sex Determination in Insects** In some insects, viz. honeybee, the male is wholly haploid and produces spermatozoa by a modified meiosis, whereby half the sperm contains the full (haploid) complement of the chromosomes and the other half contains none. The female is diploid, the queen bee produces haploid eggs, which become diploid female (workers or queen), or unfertilized eggs which develop parthenogenetically into haploid males (drones).

(v) **Sex Determination in Flowering Plants** Flowering plants are sporophyte generations and, strictly, should not be termed male and female. But this is common parlance in genetics and the end result (for example, staminate flowers, in which microspores produce male gametes) is the same. Dioecious species have separate sex chromosomes and show a pattern similar to that in mammals; the male (**staminate**) plants are XY and the female (**pistillate**) plants are XX.



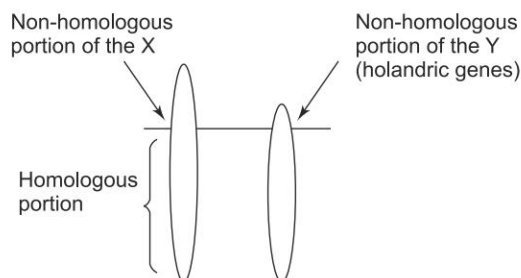
In monoecious species, e.g. maize, there are no distinct sex chromosomes; the genes that determine the development of sex organs seems to be limited throughout the chromosome complement.

Problems

45. If a sex-linked gene is a recessive lethal, causing death and reabsorption of the embryo at an early stage
 - (a) what proportion of the offspring would be expected to be female?
 - (b) and what proportion of the offspring would be expected to be carriers of the gene from a mating between two mammals, the female of which carries the gene in its heterozygous state?

46. In cats, a coat colour gene is located on the non-homologous portion of the X chromosome. BB or B— is black, bb is yellow, and Bb is tortoise shell. Are these statements true or false, give reasons.
- A mating of a black tom with a yellow tabby could give female tortoiseshell kittens.
 - A yellow male mated with a tortoiseshell female could give black male kittens.
 - The same mating can give male tortoiseshell kittens.
 - The same mating could give black female kittens.
 - A tortoiseshell tabby can have tortoiseshell kittens whatever colour tomcat she mates with.
47. A normal sighted woman whose father was red-green colourblind married a man with normal vision. What ratio of genotypes and phenotypes would be expected among their children?
48. In a marriage between two colour-blind people would all their children be colour-blind?

(vi) **Sex Linkage** In mammals the XX chromosomes of the female are homologous for their whole length but the XY chromosomes of the male are homologous for only part of their length. Both X and Y chromosomes of the male have non-homologous portions:



Since genes located in the homologous region always have an allele and behave like autosomal genes in terms of dominance and recessiveness, irrespective of sex. Genes located on the non-homologous portion of the X, however, have no allele in the male and are always manifest in the male's phenotype. Thus a recessive gene located on this portion will be manifest in the male but will be manifest in the female only when homozygous. Such a recessive gene, which remains suppressed in heterozygous condition, is known as **carrier**; 50% of the gametes would contain this gene.

Example 2.7.1

Red/green colour blindness in man is due to a recessive sex-linked gene carried on the non-homologous portion of the X chromosome. What offspring phenotypes might be expected from a marriage between a colour blind man and a normal woman whose father was colour blind?

Let X^* represent the X chromosome carrying the recessive gene for colour blindness.

(colour blind husband)	X^*Y	×	X^*Y (wife's colour blind father)
Gametes :	X^* Y		XX^* (normal wife, but heterozygous-carrier)
Offspring:	X^*X X^*X^*		$X X^*$
			XY X^*Y

Expected offspring phenotypes:

- Half the daughters normal (heterozygous or carriers)
- Half the daughters colour blind
- Half the son normal, and
- Half the sons colour blind.

Problems



49. If a woman who is heterozygous for a recessive, sex-linked gene marries a man who does not show the trait what is the probability that

- (a) the gene will be passed on to any child she may have?
- (b) she will have a child who will show the trait?
- (c) any of her sons will show the trait?
50. In man, haemophiliac (reduced ability of the blood to clot when exposed, extensive tissue bleeding following bruising, etc.) is sex-linked and recessive. What offspring phenotypic ratios would be expected from a marriage between
- (a) a haemophiliac man and a carrier heterozygous woman?
- (b) a normal man and a carrier woman?
51. What advice would a practising counsellor on genetics offer to a woman who, while having no haemophiliac symptoms herself, informed him that her father was a haemophiliac? She is married to a normal man but is concerned about the prospects of this affliction being passed on to her children.
52. The gene for the ability to taste PTU is autosomal and dominant to non-tasting. Red/green colour blindness is sex-linked and recessive. A man and his wife, both of whom can taste PTU and have normal vision, have a child who is colour blind and a non-taster.
- (a) What is the child's sex?
- (b) What is the probability (expected occurrence) of this child's phenotype in their children?
- (c) What is the probability that a child will be a taster with normal vision, like its parents?
- (d) What is the probability that a child will be a non-taster and colour blind, unlike its parent?

(vii) **Chromosome Abnormality and Sex Expression in *Drosophila*** The failure of chromosome to separate completely at Anaphase-I of meiosis can lead to the absence of the chromosome from a gamete and its presence in the diploid state in another. When such gametes are involved in fertilization with normal gametes they give rise to offspring which are, respectively, haploid and triploid with regard to that chromosome. Such meiotic failure to separate is termed **non-disjunction**, e.g. the X chromosome of *Drosophila*. When a non-disjunction, white-eyed female was crossed to red-eyed normal male produced similar abnormal offspring's, of which rare white-eyed females (XXY), XXX red-eyed super-female and XO red-eyed males were present (Fig. 2.7.2).

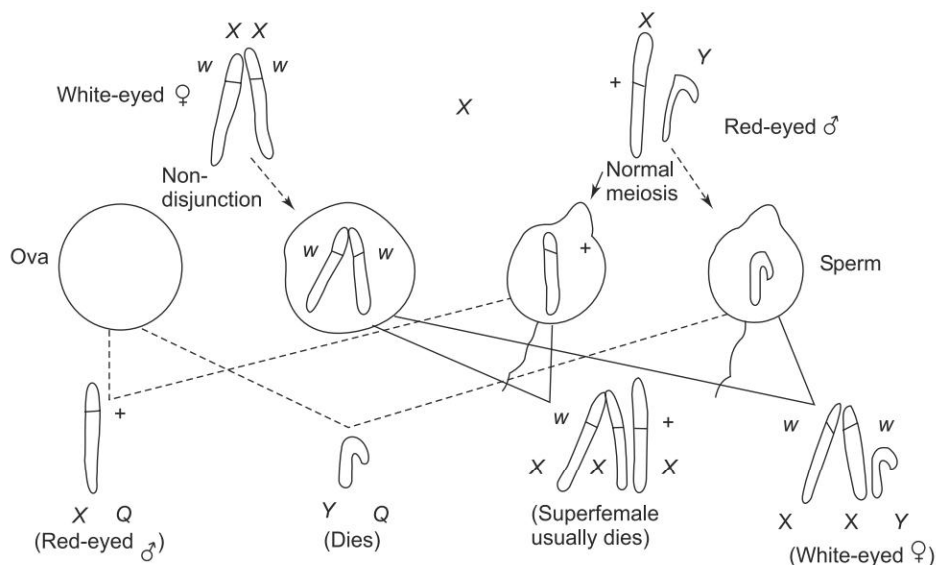


Fig. 2.7.2 Inheritance of non-disjunction X-chromosomes in *Drosophila*

In man, while XY and XX are normal male and female, respectively, XO is a sterile female with poor development of ovaries and consequently of secondary sexual characteristics, often accompanied by webbing of the neck and mental retardation (Turner syndrome). XXY is a sterile male having underdeveloped testes and little secondary sexual body hair, some mammary development (Klinefelter's syndrome (For figure see Experiment 2 in this chapter).

Problems



53. White-eye (w) in *Drosophilla* is due to a recessive, sex-linked gene. A white-eyed female was mated with a red-eyed male and the female progeny were isolated as virgins.
 - (a) What result might be expected if these females were back-crossed with their male parent?
 - (b) If, in carrying out this cross in the laboratory, some female progeny were produced which had white-eyes, what might you conclude?
54. Occasionally a male tortoiseshell cat occurs which is sterile. Suggest a possible explanation. Show, diagrammatically, a cross between a yellow tom cat and a tortoiseshell tabby which might give rise to sterility.
55. A married couple with normal colour vision have a colour blind child with Turner's syndrome.
 - (a) What is the chromosomal complement for all three individuals and their genotype with respect to the colour blindness gene?
 - (b) Construct a diagram for this cross to show where non-disjunction occurred.
56. Show diagrammatically how Turner's syndrome and Klinefelter's syndrome could arise from non-disjunction during either spermatogenesis or oogenesis.
57. (a) Show diagrammatically how chromosomes might behave in a non-disjunction sequence in oogenesis and spermatogenesis to give rise to a Mongol child.
 - (b) If the probability of a 20 year old mother having a Mongol child is 0.0003, a 40 year old mother 0.01, and a 45 year old mother 0.02, what might this indicate in relation to the answer to part (a) of this question?

(viii) **Sex-influenced Genes** These genes are autosomal but their expression in the phenotype is affected by the sex of the individual. In man, the gene for baldness acts as a dominant in the presence of male sex hormones but as a recessive in the presence of oestrogens. Gout is a painful condition caused partly by the deposition of uric acid salts in the tissues. It appears to be inherited as an autosomal dominant gene and is expressed more in men than women owing to sex differences in blood uric acid levels.

(ix) **Sex-limited Genes** These genes are also autosomal but can be expressed only in a particular sex. In cattle, genes for milk yield are limited in their expression to cows, though desirable dairy bulls would carry genes for high yield. In man, genes for male voice, body hair, and physique are autosomal, but expressed only in the presence of androgens (absent in females).

Viva Questions

- What are sex linked genes?
- A flower having female but no male reproductive parts is known as what?
- What is a hermaphrodite animal?
- What do you mean by heterogametic sex? Give examples.
- Do autosomes carry sex-determining genes?
- How is sex determined in *Drosophila*?
- Where do you find holandric gene?
- How intersexes occur in *Drosophila*?
- What is meant by carrier gene?
- How do supermales and superfemales occur in the population?
- The chance of Turner or Klinefelter's syndromes increases with the age of the female or the male parent?

**Experiment 8**

Aim Problems in Quantitative Inheritance.

Key Terminology

- **Quantitative or polygenic inheritance** The mechanism of genetic control of traits showing continuous variation.
- **Over-lapping** A group of character which extends to partly coincide with groups on either direction (or group).
- **Continuous variation** Variation measured on a continuum rather than in discrete units or categories, e.g. height, colour, etc.
- **Additive affect** A mechanism of quantitative inheritance in which alleles at different loci either add an amount to the phenotype or add nothing.
- **Phenotypic classes** Grouping on the basis of external or visual characteristic.

- **Pleiotropic effects** The phenomenon where by a single mutant affects several apparently unrelated aspects of the phenotype.

Basic Principle

In most of the inheritance pattern already discussed, had single character and has been considered as being controlled by a pair of genes at one allelic locus. However, in most organisms, a character is usually determined by more than one pair of genes on different loci of the same chromosome or different chromosomes. The Mendelian traits which were dealing so far is distinctly different from other group of traits which are known as **quantitative or metric or polygenic traits**. Some of the basic differences between qualitative and quantitative traits are as follows:

Sl.No.	QUALITATIVE TRAITS	QUALITATIVE TRAITS
1.	Known as Mendelian traits. Dominant and recessive alleles control the phenotype	Non-Mendelian traits. Only dominant alleles are active or contribute the phenotype.
2.	Limited pair of genes controls the phenotype.	Trait is governed by large number of gene pairs.
3.	Non-over-lapping characters, e.g. shape, distinct colour etc. which are easily identifiable	Over-lapping in nature, e.g. skin pigmentation, milk and fat content, weight, height, etc.
4.	Discontinuous variation, non-additive.	Continuous variation, additive in nature
5.	Traits are not influenced by environment.	Traits are very often influenced by environment.
6.	Each gene contributes distinct phenotypic effect.	Each gene contributes small amount of similar effect.

(Contd.)

7.	Phenotypic classes (or ratios) are identified by counts.	Needs scale or matrices to identify the phenotypic classes.
8.	Phenotypic classes or ratios depend on individual crosses.	Phenotypic classes depend on number of gene pairs involved in the crosses.
9.	Genes also have pleiotropic effects.	Complex pleiotropic effects exist.

Example 2.8.1

Wheat kernel, R gene makes equal contribution to redness, whereas r contributes nothing. The phenotype of all the F_1 will be intermediate between the two parents. One P_1 parent is having red kernel

while P_2 is white. But when F_2 is raised, it contains more phenotypes (and genotypes) than the F_1 .

The F_2 distribution will be similar to an expansion of the binomial, i.e. $(a + b)^4$, where, $a = R_1$, or R_2 active or contributing allele and inactive or non-contributing alleles are $b = r_1$ or r_2 and $a + b = \frac{1}{2}$ in the F_1 .

P_1 :	$R_1R_1R_2R_2$ (Red)	$\times$	$r_1r_1r_2r_2$ (White) P_2
		$\downarrow$	
F_1 :	$R_1r_1R_2r_2$ (Light red)	$\times$	$R_1r_1R_2r_2$ (Light red)
		$\downarrow$	
F_2 :	$\frac{1}{16} (R_1R_1R_2R_2)$ Red $\frac{4}{16} (2 R_1R_1R_2r_2 + 2 R_1r_1R_2R_2)$ Medium red $\frac{6}{16} (4 R_1r_1R_2r_2 + R_1R_1r_2r_2 + r_1r_1R_2R_2)$ Light red $\frac{4}{16} (2 R_1r_1r_2r_2 + 2 r_1r_1R_2r_2)$ Very light red $\frac{1}{16} (r_1r_1r_2r_2)$ white		

In this example, active or contributing alleles add an equal amount of redness to the phenotype. So the genotype $r_1r_1r_2r_2$, do not contain any R_1 or R_2 and that is why it is white.

Other characters in man, such as height and intelligence, which show a graduated pattern, are likely to be controlled by multiple genes.

shows a graduated scale in the various races of man, from Caucasian (complete white) to Negro (complete black) suggesting a multiple gene effect. Davenport (1913) analyzed the progeny of marriage between people of different skin colour. He concluded that two pairs of alleles can explain for these five categories of skin colour on the basis of the number of pigment alleles present (e.g. R_1r_1 and R_2r_2).

Example 2.8.2

Deposition of melamine skin colour in man (ignoring the effect of expression of sunlight)

P1 :	Caucasian ($R_1R_1R_2R_2$)	$\times$	$(r_1r_1r_2r_2)$ Negro		
		$\downarrow$			
F1 :	Mulato ($R_1r_1R_2r_2$)	$\times$	$(R_1r_1R_2r_2)$ Mulato		
		$\downarrow$			
No. of pigment alleles	4	3	2	1	0
Skin colour	Caucasian	Light Mulato	Mulato	Dark Mulato	Negro

The gametes produced by a light red or mulato would be in the proportion of $1aa : 2ab : 1bb$ (or $a^2 + 2ab + b^2$); which $= (a + b)^2$. Hence, the offspring genotype ratio would be shown by $\{(a + b)^2 \times (a + b)^2\}$ or $(a + b)^4 = a^4 + 4a^3b + 6a^2b^2 + 4ab^3 + b^4$.

Therefore, we can postulate with three, four or more number pairs of alleles provided they are unlinked. The Binomeal expression $(a + b)^n$ can be worked out.

Problems



58. On the basis of Davenport's findings that skin colour in man depends on the proportion of pigment genes present on two unlinked loci (two pairs of genes), and that white skin can be represented as $aaaa$ or a^4 , how would you describe the
 - (a) Black skin genotype? (b) Mulato?
 - (c) Dark mulato and (d) Light mulato?
59. Show, algebraically, the cross between
 - (a) two dark-mulato;
 - (b) two light-mulatos;
 - (c) one mulato and one dark mulato.
 For each, show the algebraic expression of their possible offspring phenotypes.
60. (a) What proportion of the children of a marriage between two light mulato parents would be expected to have white skins?
 - (b) What proportion would be expected to have the same coloured skins as their parents?
61. Assume that in the runner bean there are three unlinked loci which determine length of pod. In the short (4") pod all alleles are short (s) and in the long (10") pod all the alleles are long (l).

Each l gene contributes 1" to the length of

the pod, thus the offspring of a cross between a 4" variety ($ss\ ss\ ss$) and a 10" variety ($ll\ ll\ ll$) has pods of 7" in length ($sl\ sl\ sl$). What is the length of pod of a bean plant of the following genotypes?

(a) $ss\ ll\ ll$; (b) $ss\ ss\ ll$.

In a cross between two 7" plants, what proportion of their offspring would be expected to be:

- (c) of the 10" variety? (d) of the 7" variety?
- (e) of the 5" variety?

There are other strains of wheat with dark-red kernels (or four pairs of skin colour in man as observed by C. Stern) when crossed to white, produce F_1 phenotype intermediate between the two parents. However, only $1/64$ of the F_2 is white, and the F_1 is probably segregating for three pairs of alleles having $r_1r_1r_2r_2r_3r_3$ genotype. There can be more number of gene pairs (e.g. four or five) with more classes of kernel shades, causing difficulties in differentiating the subtle classes by eye measurement even if there is no modification of kernel colour by environment.

Solved Problem

Golden Hamburg (large breed of chicken) was crossed with Sebright Bantams (small), produce intermediate size F_1 's. Most of the F_2 population was the same as that of F_1 , whereas, some were found to exceed Golden Hamburg and some were lesser than Sebright Bantams (or **transgressive** variation i.e. offspring exceeding the size of either parental type). The contributing alleles (with capital alphabets) add equal and cumulative effects, and parents are considered to be homozygous, how can these results be explained?

Suppose for simplicity, we consider four pairs of alleles to be active.

F₁: AaBbCcDd
Intermediate (4 active alleles)

No. of active alleles	8	7	6	5	4	3	2	1	0
Phenotype	Larger than Golden Hamburg		Similar to parent		Intermediate		Similar to Parent	Smaller than Sebright Bantam	

Viva Questions



- Viva Questions**

 - How do quantitative traits differ from qualitative traits?
 - What kind of variation occurs in quantitative traits?
 - How are phenotypic classes in quantitative inheritance differentiated?
 - In quantitative traits do both the alleles contribute equal effects?
 - What is a transgressive variation?
 - If only two segregating loci contribute to cob length, and we represent the parental cross as AABB (9" cob length) $\times$ aabb (3" cob length), what will be the cob length in F_1 ?

Experiment 9

Aim Estimation of Genetic parameters and partitioning of variance.

Key Terminology

- **Genetic parameters** Measurement of genes contribution of attributes of a population (denoted by Greek letters).
- **Partitioning of variance** or **Analysis of variation** Separation of the average squared deviation about the mean of a set of data into, genetic, phenotypic and environmental components.
- **Phenotypic** Observable attributes of an organism, due to genetic and environmental interaction.
- **Genetic** Attribution of an organism which is exclusively due to genes.
- **Heritability** A measure of the degree to which the variance in the distribution of a phenotype is due to genetic causes. In the broad sense, it is measured by the total genetic variance divided by the total phenotypic variance. In the narrow sense, it is measured by the genetic variance due to additive genes divided by the total phenotypic variance.
- **Normal distribution** Any one of a family of bell-shaped frequency curves whose relative position and shapes are defined on the basis of the mean and standard deviation.
- **Standard deviation** The Square root of the variance.

Basic Principle

The last two experiments (viz. Nos. 9 and 10) have been included for Biotechnology students to learn the application of statistics in biological experiments. One should bear in mind that we take the help of statistics for biological studies not as a proof but to authenticate how our expected hypothesis closely relates to our observed data which has been generated from experiments. It helps us to draw samples from large population and test them.

In this chapter we shall learn the procedure for partitioning of phenotypic, genetic and environmental variance and also calculate heritability.

(i) The Normal Distribution Usually in the study of qualitative traits we have to consider large populations which consist of very few individuals towards extremes and more towards the nearer to the average frequency. This type of symmetrical distribution having similarity to bell-shape is known as **normal distribution**. It is approximated by binomial distribution, as because the power of binomial is very large, i.e. $(p + q)^n$. However, very often the distribution is skewed towards either direction (i.e. +ve or -ve). We will consider normal distribution in our present studies.

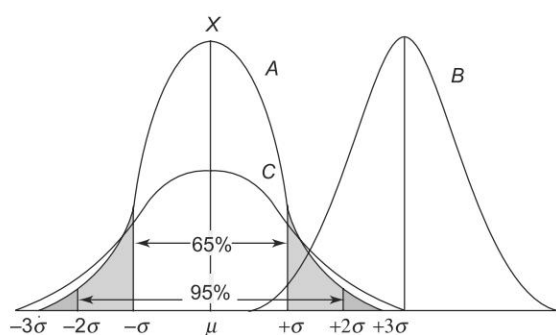


Fig. 2.9.1 Comparison of distribution curves

For calculating the arithmetic mean ($\bar{X}$ i.e. “X bar”) of average population, is given by the summation of the individual measurement (ΣX , where, Σ indicates summation of and X is

individual measurement) divided by the number of observation (N).

$$\bar{X} = \frac{X_1 + X_2 + X_3 + \dots + X_N}{N} \quad (2.9.1)$$

Generally, it is not possible to measure each and every individual in a population. Therefore, we consider a random sample out of the population for calculating $\bar{X}$. If the sample size is a true representative of the population, then the arithmetic mean will be an accurate estimation of the mean (μ) of the entire population.

(ii) Measurement of Standard Deviation In normal distribution, variables (or dispersions) may vary; therefore, it is essential to also estimate the variability along with the mean value. For genetical studies, measurement of population variability is useful, i.e.

Standard Deviation, usually represented by Greek letter “sigma” (σ). If small ‘s’ is the standard deviation of a drawn sample from a population then

$$s = \frac{\sqrt{\sum (X_1 - \bar{X})^2 + (X_2 - \bar{X})^2 + (X_3 - \bar{X})^2 + \dots + (X_n - \bar{X})^2}}{n - 1} \quad (2.9.2)$$

where, $X_1, X_2, X_3, X_4 \dots$ individual measurement, n is the sample size and the deviation (d) is squared $(X_1 - \bar{X})^2 = d^2$, summed over all individuals in the sample. A correction factor, i.e. $(n - 1)$ should be used as a denominator, if sample size is less than 30 individuals.

Usually for practical purpose, mean value (μ) is expressed along with the sample standard deviation (σ), to indicate the variability in the population i.e. $(\mu \pm \sigma)$ or extent of scattering from the mean (μ).

(iii) Variance The square of the standard deviation is called **variance** (σ^2). Standard deviation and variance can only be expressed mathematically and cannot be plotted on a normal curve. Variance is widely used in the estimation of additive genes. There are different components of

variance, viz. total **Phenotypic variance** (σ_P^2) or total expression of genetical traits in a population, which can be further partitioned into **genetic variance** (σ_G^2), non-genetic or **environmental variance** (σ_E^2), and interaction (σ_{GE}^2).

Thus,

$$(\sigma_P^2) = (\sigma_G^2) + (\sigma_E^2) + (\sigma_{GE}^2) \quad (2.9.3)$$

However, for such an analysis, it is necessary to compute “**Analysis of Variance**” (or ANOVA), which will be dealt very briefly with the help of a solved problem. Generally, to find out the genetic variance of a population either growing naturally or large numbers of cultivars are grown in a yield trial, for data collection and then analysis of variance is computed.

Solved Problem

Eight selections (including a local strain) of moong (*Phaseolus vulgaris*) numbered 1 to 8 were put under yield trial in a randomized block design (for removing soil heterogeneity). These strains were grown in equal plot size (7½ feet × 63 feet) and in six blocks. Fixed row to row and plant to plant distances were maintained in all the blocks and at maturity yield (in g) were recorded (these are necessary to reduce experimental error).

Find out the genetic parameters for these eight strains.

Following are the detail plan of the experiment, the strains sown and yield (in g) obtained from each plot.

BLOCK I		BLOCK II		BLOCK III	
YIELDS	STRAIN	YIELDS	STRAIN	YIELDS	STRAIN
4	19.5	6	27.0	3	13.5
6	25.0	2	15.0	8	13.0
3	22.0	3	21.0	2	20.5
8	11.5	7	15.0	4	19.0
7	14.5	5	22.0	7	16.0
5	21.0	8	18.0	1	15.0
1	17.5	1	20.0	5	5.5
2	27.0	4	11.0	6	12.0
2	19.5	4	24.0	2	20.5
3	24.5	7	14.0	7	14.0
7	17.5	6	31.5	6	22.0
6	29.0	2	25.0	5	24.5
5	24.5	8	16.5	3	20.5
1	31.5	3	26.0	1	21.0
8	11.0	1	20.0	8	15.5
4	19.0	5	26.0	4	16.5
BLOCK IV		BLOCK V		BLOCK VI	

For the purpose of ANOVA it is necessary to tabulate the yield according to strain and Block as given below:

BLOCK STRAIN	I	II	III	IV	V	VI	STRAIN TOTALS	STRAIN MEANS
1	17.5	20.0	15.0	31.5	20.0	21.0	125.0	20.83
2	27.0	15.0	20.5	19.5	25.0	20.5	127.5	21.25
3	22.0	21.0	13.5	24.5	26.0	20.5	127.5	21.25
4	19.5	11.0	19.0	19.0	24.0	16.5	109.0	18.17
5	21.0	22.0	5.5	24.5	26.0	24.5	123.5	20.58
6	25.0	27.0	12.0	29.0	31.5	22.0	146.5	24.42
7	14.5	15.0	16.0	17.5	14.0	14.0	91.0	15.17
8	11.5	18.0	13.0	11.0	16.5	15.5	85.5	14.25
BLOCK TOTALS	158.0	149.0	114.5	176.5	183.0	154.5	G.T.= 935.5	—

Thus from these figures we get the various sums of squares:

$$\text{Correction factor (CF)} = (935.5)^2/48 = \mathbf{18232.5}$$

$$\begin{aligned} \text{(a) The total sum of squares} &= \{(17.5)^2 + (27.0)^2 + (22.0)^2 + \dots\} - \text{CF} \\ &= 19734.8 - 18232.5 = \mathbf{1502.3} \end{aligned}$$

$$\begin{aligned} \text{(b) Sum of squares due to block} &= 1/8 \{(158.0)^2 + ((149.0)^2 + (114.5)^2 \dots\} - \text{CF} \\ &= 18598.3 - 18232.5 = \mathbf{365.8} \end{aligned}$$

$$\begin{aligned} \text{(c) Sum of squares due to strains} &= 1/6 \{(125.0)^2 + (127.5)^2 + (127.5)^2 \dots\} - \text{CF} \\ &= 18720.7 - 18232.5 = \mathbf{488.2} \end{aligned}$$

Hence, we get the sum of squares due to error by difference.

$$\text{Thus, sum of squares due to error} = \{1502.3 - (365.8 + 488.2)\} = \mathbf{648.3}$$

On the basis of the above calculation we can set down these results in ANOVA table of plot yield (in g) as below:

SOURCES OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES
Blocks	6-1=5	365.8	365.8/5 = 73.17
Strains	8-1=7	488.2	488.2/7 = 69.74
Error	{47-(5+7)} = 35	648.3	648.3/35 = 18.22
	(48 - 1) = 47	1502.3	

Thus, (from the above ANOVA table) the total phenotypic variance was partitioned into genotypic (Sources of variation due to strains) variance and environmental (Sources of variation due to error) variance. following formulae are used for computing genetic parameters:

(i) Genetic Variance

$$(\sigma_G^2) = \frac{V_S - V_E}{r} \text{ or } (\sigma_P^2) - (\sigma_E^2) \quad (2.9.4)$$

(ii) Environmental Variance

$$(\sigma_E^2) = \frac{V_E}{r} \text{ OR } (\sigma_P^2) - (\sigma_G^2) \quad (2.9.5)$$

(iii) Phenotypic Variance

$$(\sigma_P^2) = \frac{V_S}{r} \text{ OR } (\sigma_G^2) + (\sigma_E^2) \quad (2.9.6)$$

(iv) Heritability

$$(H^2) = \frac{V_S - V_E \times 100}{V_S} \text{ OR } (\sigma_G^2) \times 100 / (\sigma_P^2) \quad (2.9.7)$$

where, V_S is the mean squares due to strains, V_E is the mean squares due to error and r = number of replication. Also refer formula No. 2.9.3, which gives $(\sigma_P^2) = (\sigma_G^2) + (\sigma_E^2) + (\sigma_{GE}^2)$. Genetic variance may be further partitioned into its component to $(\sigma_A^2) + (\sigma_D^2) + (\sigma_I^2)$.

where, (σ_A^2) variance due to additive genes (or which is part of genetic variance); (σ_D^2) variance component due to complete or incomplete dominance by genes and (σ_I^2) is the genetic elements due to epistasis or interaction variance (not to be considered in this computation).

Therefore, we can calculate:

$$(\sigma_P^2) = 69.74 / 48 = \mathbf{1.453}$$

$$(\sigma_G^2) = 69.74 - 18.52/48 = \mathbf{1.067}$$

$$(\sigma_E^2) = 18.52/48 = \mathbf{0.386}$$

$$\text{or } 1.453 - 1.067 = 0.386$$

$$H^2 = 69.74 - 18.52/69.74 = \mathbf{0.7344 \text{ or } 73.44\%}$$

(iv) Heritability It is the total phenotypic variance due to additive gene effect. For improving the genetic quality of crops and livestock, it is one of the most important data. Heritability of a trait may vary from 0 to 1. As it includes all types of gene action, it is referred to as **broad sense heritability** or **broad heritability** (H^2). However, heritability,

when computed with the help of additive genetic variance divided by phenotypic variance is termed **narrow sense heritability** or **narrow heritability** and is represented as $h^2 = (\sigma_A^2)/(\sigma_P^2)$.

(Note: From the same field trial, data for other economical characters, viz. grain weight, flowering date for 50% population, plant height, insect and disease resistance, protein content, etc. collected and information of genetic component can be computed).

Some of the utility of these genetic components are:

- These genetical components are helpful in predicting the scope of crops and livestock improvement programmes.
- It can also be applied to other useful plants and wild animals in naturally living habitats. Knowledge about genetic variance and other components will also indicate about gene contribution and their scope for applying selection pressure for improvement over the existing standard.
- Variance among the population will indicate whether it is due to genes or non-genetic (environmental) components. Low genetic variance will indicate whether the species is endangered or going to be extinct or not.

Conclusion

If we carefully see into the raw data (in ANOVA layout table), minimum and maximum value of yield for plot ranges from 5.5 to 31.5, respectively. Also, strain mean values of strain Nos. 8 and 6, varies from 14.25 to 24.42 g per plot, respectively (i.e. difference of about 10.0 g per plot). This is an indication of wide variation. Moreover, Heritability value is about 73%, which is due to higher gene effect and lower environmental variance. Therefore, we may conclude from the eight selected strains of moong, that there is good scope for improving the yield.

Problems



62. In a yield trial per plot was conducted with eight high yielding cultivars of wheat in two blocks. Analysis of variance computed from the data is given in the following table.

SOURCES OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE
Block	1	5.07	5.07
Cultivars	7	230.94	32.99
Error	7	130.43	18.63
Total:	15	366.44	

- Calculate the genetic parameters and heritability (broad sense).
 - Based on the values give your comments on the scope of further improvement from these eight wheat cultivars.
63. An experiment was laid out on irrigated land to compare 12 different varieties of rice against equal doses of ammonium sulphate. These were tested in a randomized block design with six replications. Data obtained from the trial was subjected to simple analysis of variance, which is given below:

SOURCES OF VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE
Block	5	68.29	13.66
Block	11	120.03	10.91
Error	55	139.18	3.53
Total:	71	327.50	

- Calculate the heritability (broad sense).
- Give your opinion on the 12 varieties of rice against equal doses of ammonium sulphate assimilation. Would you suggest these varieties for improvement?

Viva Questions

- What is heritability (broad sense)?
- How is environmental variance related to genetic variance?
- What will be your suggestion if the heritability (in broad sense) value of a wheat population is below 40%?
- Why is Standard Deviation expressed as $\pm$, along with the mean values?
- From the breeding point of view between narrow and wide variation, which is more useful and why?
- What is the use of repetition of biological experiments?

**Experiment 10**

Aim Problems in Probability, Test of Significance (Chi-Test) and Hardy Winberg's Law.

Key Terminology

- **Probability hypothesis** The expectation of the occurrence of a particular event, based on the assumption of no experimental effect.
- **Sampling error** Biasness' occurring during data collection.
- **Observed** Based on whatever is seen or visible practically.
- **Expected** Based on theoretical assumption.
- **F₁ or F₂ ...** First offspring generation, F₁ is the first generation, and so on.
- **Null hypothesis** The statistical hypothesis that there are no differences between observed data and those data expected based on the assumption of no experimental effect.
- **Physical basis** Explained on the basis of chromosomal segregation which is visible.
- **Significance** A statistical term describing the degree to which particular results could be ascribed to chance.
- **Chi-square test** A statistical test to determine whether the observed result differs significantly from the (theoretical) expected result.

Basic Principle

The degree of uncertainty about the outcome of an experiment is greater in the biological than in the physical sciences owing to the enormous complexity of living systems. There are, however, rules to accommodate variable results and to determine the likelihood of an event's occurrence. In genetics, Mendel's Laws have remained valid to the present day. There is no 'uncertainty' regarding the segregation of alleles, or the independent assortment of unlinked alleles, since exactly 50% of daughter cells of the first meiotic division contain one member of an allelic pair. But the uncertainty arises in the formation of a zygote, as to which gamete fuses with which other. We will limit our studies on some of these statistical parameters which are very often applied to biological studies.

(i) Probability There is different types of probability studies, one of it is the probability of independent events occurring simultaneously is the **product of their individual probabilities**. Hence, if the probability of a sperm carrying t is $\frac{1}{2}$, and the probability of an ovum carrying t is likewise $\frac{1}{2}$, the probability of a zygote carrying tt is $\frac{1}{2} \times \frac{1}{2} = \frac{1}{4}$; in being independent events.

The symbol **p** (the probability, frequency, or chance that a particular event will take place)

$$p = \frac{\text{Number of favourable cases}}{\text{Total number of cases}} \quad (2.10.1)$$

Thus, if a snooker set (8 red, 6 green, 7 blue coloured balls, and 1 white ball) is placed in an otherwise empty velvet bag, the probability of picking out a white ball is:

$$p_{\text{white}} = 1/22$$

Similarly, $p_{\text{red}} = 8/22$

Thus, the probability of a certain event taking place (that a ball picked out will be a snooker ball of unspecified colour) is seen to be 22/22 or 1. Likewise an event certain not to happen (that one will pick out a croquet ball is 0/22 or 0). In other cases, probability is a fraction between 0 and 1*.

Example 2.10.1

- A. For parents who are heterozygous for the ability to taste PTU
- (a) What is the probability of the father's sperm carrying a Y chromosome?

$$p_Y = 1/2$$

- (b) What is the probability of an ovum carrying an X chromosome?

$$p_X = 1/2$$

- (c) What is the probability that a child will be a boy (XY)?

$$p_{\text{boy}} = 1/2 \times 1 = 1/2$$

- (d) What is the probability that a child will be a boy taster?

$$p_{\text{boy taster}} = 1/2 \times 3/4 = 3/8$$

- (e) What is the probability that a child will be a non-taster girl?

$$p_{\text{girl non-taster}} = 1/2 \times 1/4 = 1/8$$

- (f) What is the probability that the first child be a boy non-taster and the second a girl taster?

$$p = (1/2 \times 3/4) (1/2 \times 1/4) = 3/64$$

- B. Another type of probability is the sum rule. The probability of the occurrence of one out of a set of mutually exclusive events is the sum of their individual probabilities.

- (i) In the absence of dominance, a monohybrid cross yields a 1:2:1 offspring phenotypic ratio. For example, in a cross between a roan bull and a roan cow—

what is the probability of a calf being born either roan or white?

$$p = 1/2 + 1/4 = 3/4$$

- (ii) The polled (hornless) condition in cattle is due to a dominant gene, its recessive allele giving rise to horned. What is the probability of getting either a horned-roan or a polled-red calf from the bull and cow mentioned?

$$p = (1/4 \times 1/2) + (3/4 \times 1/4) = 5/16$$

- (iii) From the data (given above*), what is the probability of picking out either a red ball or a coloured one?

$$p = 8/22 + 13/22 = 21/22$$

Problems



64. A married couple plan to have four children. What is the probability that
- (a) The father's wish for four boys will be fulfilled?
- (b) The mother's wish for two of each sex?
- (c) The grandmother's wish for three boys and a girl?
65. A brown-eyed couple has four children, three of whom are brown-eyed and the other blue-eyed. How often would you expect this combination of offspring from such parents?
66. Assume that about 10% of Indian men have group A and that about 4/9 of these are heterozygous (AO genotype)

- (a) What is the probability that a marriage will take place between group A people?
- (b) What is the probability that a group O child will have group A parents?
67. In man there are 23 pairs of chromosomes. If possible changes in chromosome structure are ignored, what is the probability that a second child in a marriage will have the same chromosome combination as the first (i.e. will be an "identical sibling")?

(ii) The Test of Significance or Chi-square (χ^2) Test

The Mendelian ratio merely indicates the expected results from certain kinds of mating. When the number of offspring is small, observed ratios may differ quite widely from those expected. In an experiment, observed results almost always differ from those expected. This test enables the experimenter to determine to what extent the results he obtains conform to expected results, or whether the deviation from the expected results is significant. The test gives the proportion of time a particular degree of deviation is likely to occur by chance.

$$\chi^2 = \frac{\text{Sum of (Observed - Expected)}}{\text{Expected}} \text{ or } \sum \frac{d^2}{e}$$

$$= \sum_{(i)} \frac{(O - E)^2}{E_i} \quad (2.10.2)$$

where, O = Observed and E = Expected

Example 2.10.2

From a cross between a *Drosophila* known to be heterozygous for vestigial wing (vg) 100 offspring were obtained; 30 were vestigial winged and 70 normal. Is this a significant deviation from the expected result (3:1) ratio?

Phenotype	Observed (o)	Expected (e)	(o-e) = d	$d^2/e = \chi^2$
vg	30	25	+5	1 0.33
+	70	75	-5	$\chi^2 = 1.33$

From the table of Chi-square (Annexure-VI) a χ^2 of 1.33 for two classes corresponds to a chance occurrence of between 20 and 30%. In other words, in about 20 to 30% of the times this cross produced this number of offspring, this amount of deviation from the expected 3:1 ratio would be expected. This result, therefore, is not significant.

(Note: Significance may be chosen arbitrarily as 5% or less).

The test also takes into consideration the sample size and the number of variables (or degrees of freedom). All these can be included in the Chi-square (χ^2) test.

$$\chi^2 = \sum \frac{(o_1 - e_1)^2}{e_1} + \frac{(o_2 - e_2)^2}{e_2} + \frac{(o_3 - e_3)^2}{e_3} + \dots + \frac{(o_n - e_n)^2}{e_n} \quad (2.10.3)$$

However, in a sample with a small population, the Yates correction should be applied (Not to be applied for problems given below unless and until specifically mentioned).

$$\chi^2 \text{ (with correction)} = \sum \frac{[|o_1 - e_1| - 0.5]^2}{e_1} + \frac{[|o_2 - e_2| - 0.5]^2}{e_2} + \dots + \frac{[|o_n - e_n| - 0.5]^2}{e_n}$$

(Note: Chi-square test can also be applied to coin tossing experiments and concluded whether the tossing sample is to be accepted or rejected based on Chi-square values at 5% or 1%).

Problems



68. Consider the cross between two *Drosophila* known to be heterozygous for vestigial wing (vg) produced 500 flies, consisting of 150 vestigial phenotype and 350 wild type; would this deviation from the expected result be significant?
69. From a cross between a poppy plant homozygous for the recessive alleles cleft-petal and dwarf-stem (ccdd) and a plant known to be heterozygous for both alleles (+c+d) the following number of offspring phenotypes were produced:
Both characters normal – 97
Cleft-petal, normal-stem – 68
Normal-petal, dwarf-stem – 75
Cleft-petal, dwarf-stem – 80
Is this result significantly different from that expected?
70. Mendel's dihybrid cross between peas heterozygous for shape and colour ($RrYy \times RrYy$) produced the following results:
Round, yellow – 315
Round, green – 108
Wrinkled, yellow – 101
Wrinkled, green – 32
Does this result agree with the hypothesis expressed in Mendel's third law?

Application of Binomial probability distribution in genetic problems:

Example 2.10.3

Suppose, a population consists of 500 genes of which 300 are dominant and 200 are recessive. A sample of 5 is drawn at random from this population. Find the probability that

- exactly 2 genes will be dominant,
- at most 2 genes will be dominant, and
- at least 2 genes will be dominant.

Solution

We can apply Binomial probability (pr) distribution to solve this problem.

Suppose, selection of a dominant gene is considered as a success.

Therefore, the probability of success is $300/500 = 3/5 = p$; and the probability of a failure is $200/500 = 2/5 = q$

We know, $p + q = 1$

$$(a) \quad pr(x=2) = {}^5C_2 (3/5)^2 (2/5)^3 \text{ On the basis of } {}^nC_x p^x q^{n-x}$$

(where, $n = 5$; $p = 3/5$ and $q = 2/5$)

$$= \frac{5!}{2!3!} \times \frac{9}{25} \times \frac{8}{125} \\ = 10 \times \frac{9}{25} \times \frac{8}{125} = 144/625 \\ = 0.230 \text{ (approx)} < 1.$$

$$(b) \quad pr(x \leq 2) = p(x=0) + pr(x=1) + pr(x=2) \\ = {}^5C_0 (2/5)^5 + {}^5C_1 (3/5)^1 (2/5)^4 \\ + {}^5C_2 (3/5)^2 (2/5)^3 \\ = \{1 \times (32/3125) + (5 \times 3/5 \times 16/625) + (10 \times 9/25 \times 8/125)\} \\ = 32/3125 + 48/625 + 144/625 \\ = (32 + 240 + 720)/3125 = 992/3125 \\ = 0.317 \text{ (approx.)} \leq 1.$$

$$(c) \quad pr(x \geq 2) = 1 - [p(x=0) + pr(x=1)] \\ = 1 - {}^5C_0 [(2/5)^5 + {}^5C_1 (3/5)^1 (2/5)^4] \\ = 1 - (32/3125 + 48/625) \\ = 1 - 0.087 = 0.913 \text{ (approx.)}$$

(iii) Hardy Winberg's Law The genetic situations so far discussed in this chapter have involved the pattern of inheritance in particular mating or in plant crosses or families. But in the main, the utility of Mendelian theory lies in its applicability to population, since herein lies its use in social medicine, in agriculture, and in our understanding of the mechanism of evolution. If evolution is seen in terms of changes in the frequencies of the genes that make up the **gene pool** (the total complement of genes in the population which can be passed on to the next generation) the patterns of gene distribution within a population must be understood.

The distribution of alleles in a randomly mating population was worked out independently by Hardy in England and Weinberg in Germany, in 1908. By a randomly mating population the geneticist means a population in which a particular combination of alleles does not render an individual more or less likely to achieve mating than any other combination. Moreover, the population must not be subject to mutation, natural selection pressure, or the immigration of genes from surrounding population.

According to the Binomial distribution, when two independent events are occurring with the probability p and q , then their combined probability of joint occurrence will be the product of the independent events, i.e. pq . That is to say, occurrence and non-occurrence of any one does not affect the probability of occurrence of any of the other. If this independent event is represented for a number (infinite times) of times of trials, the sum of the factors is the Binomial must add to unity, i.e. $p + q = 1$. For example, a coin may settle in four ways:

1st: Heads (p) and Heads (p) = p^2

2nd: Heads (p) and Tails (q) = pq

3rd: Tails (q) and Heads (p) = qp

and 4th: Tails (q) and Tails (q) = q^2

which may also be expressed as: $p^2 + 2pq + q^2 = 1.0$. If we now consider a pair of alleles, A and a, the F_2 generation's alleles can be described by the expression of the Binomial, and called the frequency (proportion) of the A allele p and the frequency of the a allele q , then the probability of an organism having both A genes is pp or p^2 ; likewise for an organism having aa it is qq or q^2 . Now, if the coin is tossed three times, the probability for heads and tails will be found by multiplying, i.e. $(p^2 + 2pq + q^2)(p + q) = p^3 + 3p^2q + 3pq^2 + q^3$. Thus, we can find the expanded Binomial by raising its power n to any number of times, say infinite.

Solved Problems

1. In a randomly breeding population of mice, agouti coat (A) is dominant to non-agouti (a). In a

sample 16% were found to have non-agouti coat.

- (a) What are the frequencies of the agouti alleles in the population? Let the frequency of the agouti gene be p and the non-agouti gene be q .

The non-agouti animals are genotype aa (or q^2) which is 16% of the population.

$q^2 = 16\%$ or 0.16. Therefore, $q = 0.4$ (the frequency of the a allele) and, since $p + q = 1$, $p = 0.6$ (the frequency of the A allele).

- (b) What proportion of the population would be expected to be homozygous for A and what proportion heterozygous?

From $p^2 + 2pq + q^2$,

$$p^2 = (0.6)^2$$

= 0.36, or 36% of the population is AA

$$2pq = 2(0.6)(0.4)$$

= 0.48, or 48% of the population is Aa.

(Note: Since $p + q = 1$, then $(p + q)^2 = 1$, and $p^2 + 2pq + q^2 = 1$, as shown by this example).

- (c) If the population continues to breed randomly what is the distribution of genes in the next generation?

The proportion of A-carrying gametes (all those produced by the AA animals and half of those produced by the Aa animals)

$$= 0.36 + \frac{1}{2}(0.48) = 0.6$$

The proportion of a-carrying gametes (half the Aa animals and all those produced by the aa animals)

$$= \frac{1}{2}(0.48) + 0.16 = 0.4$$

The distribution in the following generation

$$= (0.6A + 0.4a)^2$$

$$= 0.36AA + 2 \times 0.24Aa = 0.16aa,$$

which is the same proportion of genotypes as in the preceding generation.

2. If the frequency of a third allele is termed r , then $p + q + r = 1$ and the distribution of alleles is given, as before, by $(p + q + r)^2$.

If the frequency of ABO blood group alleles in a certain population was known to be:

$$I^A \text{ 20\%; } I^B \text{ 10\%; and } i \text{ 70\%,}$$

what would be the expected proportion of blood groups in the population?

Let the frequency of I^A , I^B and i be termed p , q , and r respectively.

Then the frequency of genotypes is given by

$$(p + q + r)^2 = p^2 + q^2 + r^2 + 2pq + 2pr + 2qr$$

Genotype	$I^A I^A$	$I^B I^B$	ii	$I^A I^B$	$I^A i$	$I^B i$
Group	A	B	O	AB	A	B
Group A frequency	$= p^2 + 2pr$					

$$= (0.2)^2 + 2(0.2)(0.7)$$

$$= 0.32, \text{ or } 32\%;$$

$$\text{Group B frequency} = q^2 + 2qr$$

$$= (0.1)^2 + 2(0.1)(0.7)$$

$$= 0.15 \text{ or } 15\%;$$

$$\text{Group O frequency} = r^2$$

$$= (0.7)^2$$

$$= 0.49 \text{ or } 49\%;$$

$$\text{Group AB frequency} = 2pq$$

$$= 2(0.2)(0.1)$$

$$= 0.04, \text{ or } 4\%$$

Clearly the series can be extended to accommodate a greater set when

$$p + q + r \dots \text{etc.} = 1$$

In conclusion, the Hardy-Weinberg principle holds that the relative frequency of alleles remain constant from generation to generation, as do the genotypic ratios. It should be stressed that the principle refers to randomly breeding population devoid of natural selection pressure and changes in gene content due to mutation or exchange with neighbouring populations. Thus its application in an untreated form to evolving natural populations could lead to error.

Problems



71. The gene for ability to roll the tongue in man is dominant (R) to non-rolling (r), and not sex-linked.
If, in a given sample of the population, the proportion of the different genotypes were: $\frac{1}{4} RR : \frac{1}{2} Rr : \frac{1}{4} rr$,
Would this ratio be the same in both sexes?
What would be the expected proportion of sperm carrying R and r genes?
What would be the expected proportion of sperm carrying R and r ova?
72. In a class of 25 students tested for the ability to taste (T) Phenyl Thiourea (PTU), nine were found to be non-tasters (t).
(a) What is the frequency of the t allele?
(b) What is the frequency of the T allele?
(c) About how many of the sixteen tasters would you expect to be heterozygous?
73. A farmer bought a herd of 600 sheep, taken from a freely breeding population and later found that 150 of the animals had an economically undesirable feature, crinkly-hair, caused by the recessive gene (cr).
(a) What is the frequency of this undesirable allele in the herd?
(b) What proportion of the herd is likely to be heterozygous?
74. A packet of *Nicotiana* seeds was bought from a seed man and, when the seeds germinated, 192 seedlings were albino and 2880 were green.
(a) What proportion of the seedlings would be expected to be homozygous for chlorophyll production?
(b) What proportion of the seedlings would be expected to be carriers of the albino gene?
75. In a sample of 2400 births at a maternity hospital, six babies died shortly after birth from the effects of a recessive gene (co) for colonic obstruction.
(a) What is the frequency of this gene in the population?
(a) What proportion of the population is heterozygous and what proportion is homozygous for the normal allele?
76. If, in man, a recessive lethal gene has a frequency in the population of 0.03, about how many deaths attributable to the effects of this gene would be likely to occur in 10,000 births?

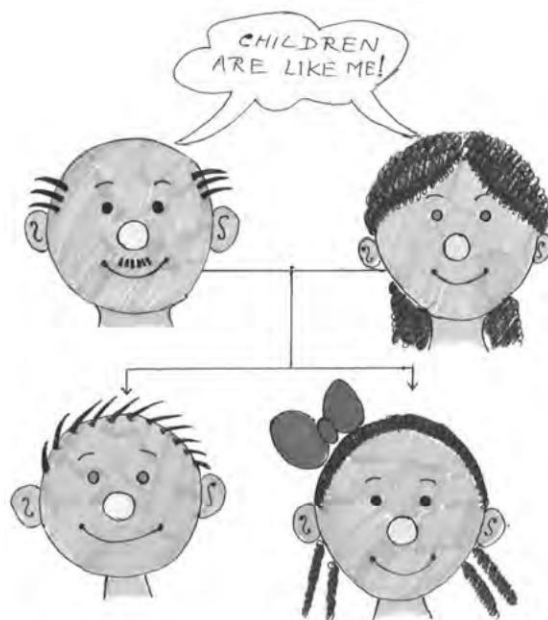
Viva Questions



- Hardy-Weinberg rule is an important model for what type of study?
- What is a gene pool?
- What information do we get from Chi-square values?
- What is null-hypothesis?
- What is meant by the significance and non-significance of Chi-square values?
- Why are biological events based on 50 : 50 probabilities?

Notes and Sketches





3

Microbiology



Experiment 1A

Aim Isolation of bacteria from Curd, Root nodules, Soil and Water samples.

Key Terminology

- **Colony** A viable mass of microorganisms growing on an agar surface, usually originating from a single organism.
- **Microorganism** Microscopic or sub-microscopic organism, viz. bacterium, virus, or fungus.
- **Pure culture** All organisms which are descendents of the same or single organism.
- **Streaking** Move or rub the inoculating loop very fast in a zigzag fashion on agar surface, so that organisms are separated and deposited on the agar to develop a colony.
- **Streak plate** Common method of separating bacterial cells on the agar surface to obtain isolated single colonies.
- **Pour plate** Method for obtaining bacterial colonies. The bacteria are mixed with melted agar until evenly distributed and poured into an empty petri plate for solidification.
- **Spin plate** Method for isolating bacteria. Diluted bacteria are pipetted onto the agar surface and with the help of bent glass rod it is evenly distributed over the entire agar surface.
- **Isolated plate** After incubation, single colonies that appear separated and far enough apart on a agar surface.
- **Discrete colonies** The area near the end of the streaking colony, shows a pattern which

separates and is distinctly different from the rest.

- **Selective media** A media that inhibit the growth of one group of organisms while permitting the growth of another organism.
- **Differential media** A media that cause an observable colour change in the medium when a particular chemical reaction occurs.
- **Incubation** To keep bacterial cells at a suitable temperature so that they develop or multiply.
- **Media enrichment** Special additives for the medium that enhance the growth of certain organisms.
- **Selective combination** A combination of selective and differential media that permit the growth of one group of organism while inhibiting the growth of another.
- **Enteric bacilli** Group of bacteria occurring in the intestines.

Basic Principles

Microorganisms exist in nature, viz. curd, root nodules, soil or water as mixed population. However, to study microorganisms in the laboratory we must have them in the form of a **pure culture**, that is one in which **all organisms are descendant of the same organism**. When microorganisms are cultured, it develops a **colony**. A colony is a visible mass of microorganisms growing on an agar surface and usually originating from a single organism or arrangement of organism.

There are **four** different methods of isolating bacteria from curd, root nodules, soil or water:

(i) Streak Plate Method of Isolation: The most common way of separating bacterial cells on the agar surface to obtain isolated colonies is the streak plate method. It provides a simple and rapid method of diluting the sample by mechanical means. As the loop is streaked across the agar surface, more and more bacteria are rubbed off until individual separated organisms are deposited on the agar. After incubation, the area at the beginning of the streak pattern will show confluent growth, while the area near the end of the pattern should show discrete colonies.

(ii) The Pour Plate Method of Isolation: Another method of separating bacteria is the pour plate method. With the pour plate method, the bacteria are mixed with melted agar until evenly distributed and separated throughout the liquid. The melted agar is then poured into an empty plate and allowed to solidify. After incubation, discrete bacterial colonies can be found growing both on the agar and in the agar.

(iii) The Spin Plate Method of Isolation: It involves diluting the bacterial sample in tubes of sterile water, saline, or broth. Small samples of the dilute bacteria are then pipetted onto the surface of agar plates. A sterile, bent-glass rod is then used to spread the bacteria evenly over the entire agar surface in order to see isolated colonies.

(iv) Use of Specialized Media of Isolation: To supplement mechanical techniques of isolation such as streak method, many **special-purpose media** are available to the microbiologist to aid the isolation and identification of specific microorganisms at the same time. These special purpose media fall into four groups:

(a) Selective media—A selective medium has agents which will **inhibit the growth of one group of organisms while permitting the growth of another**, e.g. **Columbia CAN agar** has the antibiotic colistin and nalidixic acid added to it which inhibit the growth of gram-negative bacteria but not the growth of gram-positives.

(b) Differential media—It contains additives that **cause an observable colour change in the medium when a particular chemical reaction occurs**. They are useful in differentiating bacteria according to some biochemical characteristic. In other words, **they indicate whether or not a certain organism can carry out a specific biochemical reaction** during its normal metabolism.

(c) Enrichment media—It contains additives that **enhance the growth of certain organisms**. This is useful when the organism you wish to culture is present in relatively small numbers compared to other organisms growing in the mixture.

(d) Combination selective and differential media—It permits the growth of one group of organisms while inhibiting the growth of another. In addition, it differentiates those organisms that grow based on whether they can **carry out particular chemical reactions**, e.g. eosin methylene blue (EMB) agar is selective for gram-negative bacteria. The dyes eosin Y and methylene blue found in the medium inhibit the growth of gram-positive bacteria but not the growth of gram-negative. In addition, it is useful in differentiating the various gram-negative enteric bacilli belonging to the bacterial family *Enterobacteriaceae*.

There are literally hundreds of special-purpose media available to the microbiologist. In this experiment we will learn streaking and spin plate methods of isolating bacteria. It may be noted that the first three experiments are somewhat interrelated.

Materials

- Sample of curd or root nodules or soil or water (Any other materials may be selected)
- 1 ml pipettes, 0.1 ml pipettes (sterilized)
- Inoculating needle and glass-spreader,
- Petri-plates, culture tubes or 100 ml widemouthed conical flasks containing each of the following media,
 - a. Tryptic soy agar (*See Annexure III, No.1*)
 - b. Nutrient agar acetone (*See Annexure III No. 9*)

c. EMB Agar (See Annexure III No. 12)

- Incubator set at 37°C
- Sterilized dilution blank bottles
- Sterilized water or saline water
- Vortex
- Glass marking pencil

Methods

1. Take 1 g curd or a few root nodules or 1 g soil or 1 ml of water and add it to a dilute blank bottle containing 9 ml sterilized saline or distilled water. (Before adding the root nodules, sterilize the surface and crush them in 1 ml of sterilized water). Vortex the mixture thoroughly. This gives a dilution of 1:10 or 10^{-1} .
2. From the above mixture, transfer 1 ml of the suspension to another dilution blank bottle containing 9 ml of sterilized saline or distilled water using a 1 ml sterilized pipette. Vortex it thoroughly. This will be 1:100 or 10^{-2} dilution.
3. **Repeat Step 2** to prepare dilution of 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and so on.
4. From each dilution, pipette 0.1 ml of the sample and spread it on agar (prepared in plates) using the glass-spreader. The mixture must be diluted until the various **individual microorganisms become separated** far enough apart on the agar surface that after incubation they form visible **colonies isolated from the colonies of other microorganisms**. This plate is called an **isolated plate**.
This will also indicate the correct dilution needed for isolation.
5. For streaking method lift the edge of the lid enough to insert the inoculating loop. Streak

the loop across the surface of the agar medium using either the pattern shown in Fig. 3.1.1. In order to avoid digging into the agar as you streak the loop over the top of the agar you must keep the loop parallel to the agar surface. Always start streaking at the “12 o’clock position” of the plate and streak side-to-side as you pull the loop toward you.



Fig. 3.1.1 Different pattern of streaking

6. Incubate the plate at 37°C in an incubator for 24 h. Study the colonies and their morphological feature.

NOTE: See Annexure III for Stains and Media preparations.

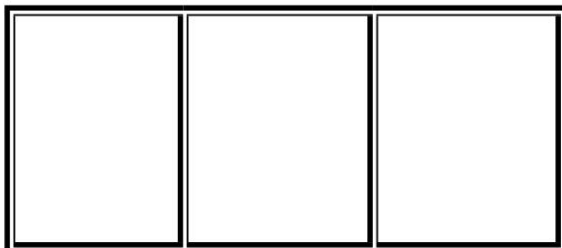
Observations

Observe isolated colonies on the petri-plate and record your observations. A large number of white or off-white colonies with smooth, rough or gummy surface may appear. Different microorganisms will frequently produce colonies which differ in their morphological appearance, e.g. elevation, form, margin, surface, optical characteristics and pigmentation.

Colony No.	Elevation	Form	Margin	Surface	Optical characteristics	Pigmentation
#1						
#2						
#3						
#4						

Figures

Draw the pattern of colonies on your practical record.

**Precautions**

- Each time you use the loop do not forget to flame and cool the loop in between two inoculations.

Viva Questions

- Which bacteria are present in milk?
- What is the principle of curd making?
- What is the morphology of bacteria isolated from root nodules?
- What is a colony?

**Experiment 1B****Aim**

Isolation of bacterial and fungal spores from 'Air samples'.

Key Terminology

- **Dissemination** Circulation or spreading of air particles or pathogens.
- **Transmission** The process of being carried from one place to another.
- **Samplers** Readymade devices available for collecting air samples.

Basic Principle

The aim of this field of study is to compare the bacterial and fungal occurrences in and around our environment. Air-control measures are crucial for reducing **disseminations** of airborne biological particles in different environments, e.g. stratosphere, laboratory, toilet, hospital, etc. different environmental sites are expected to have different **densities of airborne microflora** which are responsible for **transmission of pathogens**. Routine environmental monitoring programme can help to avert epidemics, housing high-risk

Never lay the loop down once it is sterilized. It may again become contaminated.

- When picking off single colonies, remove the top portion of the colony without touching the agar surface to avoid picking up any inhibited bacteria from the surface of the agar.
- For plating the dilution on nutrient agar, start from the highest dilution.
- Along with dilution method also use the streak plate method of isolation for obtaining isolated colonies from a mixture of microorganisms.
- Use Glass-marking pencil for labelling all Culture plates or tubes etc.
- If the agar surface of the petri-plate is visibly wet, use a sterile swab to gently remove the water.

patients in a hospital, and to detect appearance of any new airborne pathogens.

There are various **samplers** for collecting 'air samples', e.g. 4-sieve impactor air samplers, Sampler MK2, Air Ideal (bio Merieux), SAS Super 100 (with 1 sampling head, Fisher Bio-block), and Air Sampler Mas-100 (Merck), etc. However, in this experiment we are going to study a simpler form of collecting bacterial and fungal spores from three different sampling sites (or locations), two growth media, viz. Tryptic soy agar and malt extract agar for three different durations.

Materials

- ∞ 20 petri-plates {2 control + (3 locations × 2 media × 3 durations)} (1 + 9) of which containing each of the following media:
 - (a) Trypticase soy agar (TSA) (*See Annexure III, No.1*)
 - (b) Malt extract agar (MEA) (*See Annexure III, No. 19*)

- Incubator set at 30° and 37°C
- Glass marking pencil

Methods

The protocol for isolation of bacterial and fungal spores from 'Air samples' of laboratory (L_1), toilet (L_2) and hospital (L_3) is as follows:

1. Take all the 20 petri-plates (9 with TSA + 9 MEA + 2 controls) and mark them with a glass marker mentioning what type of media (preferably Sl. No.) or the petri-plate cover, which will remain uncovered, viz. for 1 h (T_1), 2 h (T_2) and 4 h (T_3) duration.
2. Remove the cover and place 3 petri-plates each in a suitable place (place 8 m apart to avoid interference) or any disturbance in three locations, viz. L_1 , L_2 and L_3 .
3. After 1 h (or T_1) replace the covers of six petri-plates (2 each from L_1 , L_2 and L_3) × (TSA and MEA). Repeat it for 2 h and 4 h also.
4. Incubate all the plates (including control) in an incubator. TSA petri-plates were incubated for 3 days at 37°C to detect bacterial growth and MEA for a further 5 days at 30°C to detect fungal growth.
5. Bacterial colonies were counted on day 1 and day 5 (despite the possibility that bacteria can inhibit fungal growth).

Note: This experiment may be further extended by isolating single colonies from different bacterial and fungal colonies obtained from different 'Air samples' locations and classify them into gram positive as well as gram negative bacteria and identify the fungus.

Observations

Examine the obtained colonies from the laboratory (L_1), toilet (L_2) and hospital (L_3) at different durations (viz. T_1 , T_2 and T_3) and record your observations in the Result Table given below. A number of white or off-white colonies with smooth, rough or gummy surface may appear. Colony elevation, form, margin and pigment characteristics are to be recorded.

Morphological appearance of fungus may differ in structure and forms. Different microorganisms appearing in different location, as well as periods may be recorded.

Describe the type of microorganisms or pattern appearing in one location which may differ from the other. Moreover, concentration of colonies may also differ with durations.

Results may be expressed (for each combination) as number of bacterial or fungal colonies appearing per unit time per unit area of petri-plate (diameter)

Results

Record your result in the following table*:

Location	Durations	Media Sl. No.	Description of bacterial colonies	Description of fungal colonies
Control	0 h	TSA 1	No colony	No colony
		MEA 2	No colony	No colony
Laboratory(L_1)	1 h (T_1)	TSA 3		
		MEA 4		
	2 h (T_2)	TSA 5		
		MEA 6		
	4 h (T_3)	TSA 7		
		MEA 8		

* Similar tables may be drawn for toilet (L_2) and hospital (L_3)

Precautions

- Use a glass marking pencil for labelling the petri-plates' covers, indicating the treatment combination.
- During incubation put the petri-plates upside down in the incubator.
- Caution should be maintained while covering the petri-plates with respective treatment combination (Keep the cover attached with the respective media plate).
- Petri-plates are to be placed in such a location, so that they are not disturbed during exposure.

Viva Questions

- How do the types of bacterial and fungal colonies differ with locations and durations?
- How can you utilize these informations (or data) for practical benefit of human beings?
- What do you expect, if 'air samples' are collected from different levels of the stratosphere?
- What measures should you take to control or reduce the concentration of bacterial and fungal colonies in a particular site, e.g. toilet or laboratory?

**Experiment 2****Aim**

Aseptic technique and transfer of microorganisms for pure culture.

Key Terminology

- **Sterile nutrient** Containing a medium in which only the desired microorganism can be grown.
- **Sterile medium** Free of all life forms.
- **Medium** Anything on or in which microorganisms are grown.
- **Inoculum** A pure culture of microorganism used for transforming in sterile medium without having any unwanted outside contaminants.
- **Aseptic technique** Preventive measures taken to check unwanted microorganisms from gaining access in the medium.
- **Incinerated** Destroy by burning.
- **Plate culture** Petri plate in which the inoculum exists or microorganism is grown.

Basic Principle

In natural environments, microorganisms usually exist as mixed populations. However, if we are to study, characterize, and identify microorganisms, we must have the organisms in the form of a **pure culture**. A pure culture is one in which all organisms are descendants of the same organism. Special technique is applied to obtain pure culture. In working with microorganisms we must have a

sterile nutrient-containing-medium in which to grow the organisms. Anything in or on which we grow a microorganism is termed a "medium". A sterile medium is one which is free of all life forms. It is usually sterilized by heating it to a temperature at which all contaminating microorganisms are destroyed.

Finally, in working with microorganisms, we must have a method of transferring growing organisms called **inoculum** that form a pure culture to a sterile medium without introducing any unwanted outside contaminants. This method of preventing unwanted microorganisms from gaining access is termed "aseptic technique". For all future lab work one should start with aseptic technique.

Materials

- Inoculating needle and glass-spreader
- Petri-plates, culture tubes or 100 ml wide-mouthed conical flasks containing media, viz. Tryptic soy agar (See Annexure III, No. 1)
- Incubator set at 37°C
- Glass marking pencil
- Inoculum (Organisms growing in plate or tube or flask).

Methods

The procedure for aseptically transferring micro-organisms is as follows:

1. The inoculating loop is sterilized by passing it at an angle through the flame of a spirit lamp or gas burner until the entire length of the wire becomes orange from the heat and all contaminants on the wire are incinerated.



Fig. 3.2.1 Demonstration—how to hold the culture tube (in the left hand) and inoculating the needle/loop and plug/cap in the right hand

(a) Raising Inoculum from Culture Tube

2. For removing the inoculum, hold the culture tube in one hand and in your other hand, hold the sterilized inoculating loop as it were a pencil. Remove the cap or plug of the pure culture tube with the little finger of your loop hand (Fig. 3.2.1) (never lay the cap or plug down).



3. Very briefly flame the lip of the culture tube. This creates a convection current which forces air out of the tube and preventing air-borne contaminants from entering the tube.
4. Keeping the **culture tube** at an angle, insert the inoculating loop and remove a loopful of inoculum (Fig. 3.2.2). Again flame the lip of the culture tube and replace the cap or plug.
5. For removing the inoculum from **plate culture**, lift the lid of the plate slightly and stab the sterilized loop into the agar away from any growth to cool the loop. Then scrape off a small amount of the organisms and close the lid.

(b) Incubation

6. To transfer the inoculum to the sterile medium, pick up the sterile broth tube and remove the cap or plug with the little finger of your loop hand. Flame the lip of the broth tube and place the loopful of inoculum into the broth.

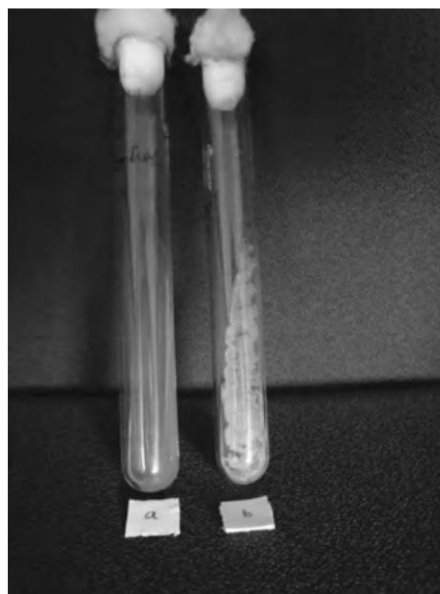


Fig. 3.2.2 Procedure for inoculating culture tube with inoculating needle/loop

7. Withdraw the loop. Again flame the lip of the tube and replace the cap or plug. Resterilize the loop before you put it down.
8. If you want to transfer the inoculum into a petri-plate, lift the edge of the lid just enough to insert the loop and follow Step 5 in Experiment 1.
- 9 Place the culture tubes or petri plates or broth culture in an incubator maintained at 37°C for 18–24 h.

Observation and Results

Draw and describe the growth seen in the cultures. Recognize and identify the following types of growth in the petri-plate or broth culture: pellicle,

turbidity sediment, and any combination of these. State the colour and pigmentation if any seen on the plate cultures.

Using an inoculating loop, explain how to aseptically remove some inoculum from either a broth tube, slant tube or petri-plate and inoculate a sterile broth tube, slant tube, or petri-plate without introducing outside contamination.

Precautions

- Dispose of all materials when the experiment is completed.
- Remove all markings from the glasswares, properly clean them and place them in their designated places.

Viva Questions

- Define the following terms: Sterile medium, inoculum, aseptic technique, and colony.
- What are the three types of growth that may be seen in a broth culture?
- What does each colony represent?



Experiment 3A

Aim

Staining Gram Positive (+ve) and Gram Negative (–ve) bacteria.

Key Terminology

- **Gram stain** Purple or pink stain developed by bacteria when crystal violet-iodine complex.
- **Differential stain** Staining procedure used to differentiate bacteria.
- **Gram positive** Bacteria that stain purple colour with the gram staining procedure.
- **Gram negative** Bacteria that stain pink colour with the gram staining procedure.
- **Osmotic lysis** Flow of water inside or outside the cell wall, which confers cell rigidity.
- **Basic dye (Crystal-violet)** Crystal violet-iodine complex used to stain bacteria.
- **Gram's decolourizer** A mixture of ethyl alcohol and acetone, which helps gram-positive bacteria to retain this complex, whereas gram-negative are decolourized.

Basic Principle

Gram stain is the most widely used staining procedure in bacteriology. It is called a “differential stain” since it differentiates between gram-positive and gram-negative bacteria. Bacteria that stain purple with the gram staining procedure are termed gram-positive; those that stain pink are said to be gram-negative.

Gram-positive and gram-negative bacteria stain differently because of fundamental differences in the structure of their cell walls. The bacterial cell wall serves to give the organism its size and shape as well as to prevent osmotic lysis. The material in the bacterial cell wall which confers rigidity is called **peptidoglycan**.

(i) Gram-positive In electron micrographs, the gram-positive cell wall appears a broad, dense wall 20–80 nm thick and consisting of

numerous interconnecting layers of peptidoglycan. Chemically, 60 to 90% of the gram-positive cell wall is peptidoglycan. Interwoven in the cell wall of gram-positive are teichoic acids. Teichoic acids, which extend through and beyond the rest of the cell wall, are composed of polymers of glycerol, phosphates, and the sugar alcohol ribitol. Some have a lipid attached (lipoteichoic acid). The outer surface of the peptidoglycan is studded with proteins that differ with the stain and species of the bacterium.

(ii) Gram-negative On the other hand, gram-negative cell wall contains only 2–3 layers of peptidoglycan and is surrounded by an outer membrane composed of phospholipids, lipopolysaccharide, lipoprotein, and proteins. Only 10–20% of the gram-negative cell wall is peptidoglycan. The phospholipids are located mainly in the inner layer of the outer membrane, as are the lipoproteins that connect the outer membrane to the peptidoglycan. The lipopolysaccharides, located in the outer layer of the outer membrane, consist of a lipid portion called **lipid** embedded in the membrane and a polysaccharide portion extending outward from the bacterial surface. The outer membrane also contains a number of proteins that differ with the stain and species of the bacterium.

With the current theory behind gram staining, it is thought that in gram-positive bacteria, the crystal violet and iodine combine to form a larger molecule that precipitate out within the cell. The alcohol/acetone mixture then causes dehydration of the multilayered peptidoglycan, thus decreasing the space between the molecules and causing the cell wall to trap the crystal violet-iodine complex within the cell. In the case of gram-negative bacteria, the alcohol/acetone mixture, being a lipid solvent, dissolves the outer membrane of the cell wall and may also damage the cytoplasmic membrane to which the peptidoglycan is attached. The single thin layer of peptidoglycan is unable to retain the crystal violet-iodine complex and the cell is decolourized.

Materials

- Gram's iodine solution (*See Annexure III*)
- Crystal violet-iodine stain (*See Annexure III*)
- Safrinin stain (*See Annexure III*)
- Inoculating needle and glass-spreader
- Inoculum of gram-positive and gram-negative bacteria, e.g. *Escherichia coli* and *Staphylococcus epidermidis*.
- Ethyle alcohol and acetone
- Distilled water in wash-bottle
- Slides and cover slips.
- Microscope (with oil immersion).

Methods

The gram-staining procedure involves four steps

1. The bacteria are first stained with the basic dye crystal-violet. Both gram-positive and gram-negative bacteria become directly stained and appear purple after this step.
2. The bacteria are then treated with gram's iodine solution. This allows the stain to be retained better by forming an insoluble crystal violet-iodine complex. Both gram-positive and gram-negative bacteria remain purple after this step.
3. Gram's decolourizer, a mixture of ethyle alcohol and acetone, is then added. This is the differential step. Gram-positive bacteria retain the crystal violet-iodine complex while gram-negative bacteria get decolourized.
4. Finally, the counter stain saffranin (also a basic dye) is applied. Since the gram-positive bacteria are already stained purple, they are not affected by the counter stain. Gram-negative bacteria, which are now colourless, become directly stained by the saffranin. Thus, gram-positive bacteria appear purple and the gram-negative bacteria appear pink.
5. Heat fix a smear of *Escherichia coli* as follows:
 - (a) Using the dropper bottle of distilled water, place half of a normal sized drop

- of water on a clean slide by touching the dropper to the slide.
- (b) Aseptically remove a small amount of *E. coli* from the agar surface and just touch it several times to the drop of water until it just turns cloudy. Flame the loop and let it cool.
 - (c) Using the loop spread the bacteria-water mixture over the entire slide to form a thin film.
 - (d) Allow this thin suspension to completely air dry.
 - (e) Pass about one inch of the slide (film side up) through the flame of the burner several times until it just becomes uncomfortable to hold in order to heat-fix the bacteria to the slide.
6. Stain with crystal-violet for one minute. Gently wash with water. Shake off the excess water but do not blot dry between steps.
 7. Stain with gram's iodine solution for one minute and gently wash with water.
 8. Decolourize by adding gram's decolourizer drop by drop until the purple stops flowing. Wash immediately with water.
 9. Stain with safrinin for two minutes and wash with water.
 10. Blot dry and observe using an oil immersion microscope.
 11. Make a second gram stain using a plate of *Staphylococcus epidermidis* and repeat Steps 5 to 11.

Observations

Determine if a bacterium is gram-positive or gram-negative when observed under a microscope after staining and state the arrangement of the organism.

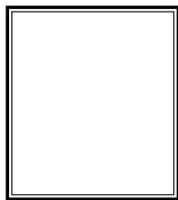
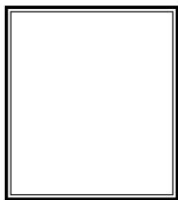
Viva Questions



- What is the basis of gram's staining?
- Why is the gram stain said to be a differential stain?
- What are the basic differences in gram-positive and gram-negative cell wall in bacteria?

Results

Make drawings of each bacterium on your gram stain preparations.

	
Gram stain of <i>E. coli</i>	Gram stain of <i>Staphylococcus epidermidis</i>
Colour: Gram reaction: Shape:	Colour: Gram reaction: Shape:

Precautions

- It is important to note that gram-positive (the ability to retain the purple crystal violet-iodine complex) is not a regular phenomenon but a matter of degree. There are **several factors that could result in a gram-positive organism staining gram negatively.**
 - (a) Overheating during heat fixation, over decolourization with alcohol, and even too much washing with water between steps may result in gram-positive bacteria losing the violet-iodine complex.
 - (b) The age of the culture. Culture more than 24 h old may then lose ability to retain the crystal violet-iodine complex.
 - (c) Some gram-positive bacteria are more able to retain the crystal violet-iodine complex than others.

Therefore, one must use very precise techniques in gram staining and interpret the result with discretion.

- Also see *Experiment 1*.

- Why can gram-positive bacteria retain the crystal violet-iodine complex while gram-negative bacteria become decolourized?
- What is the clear area on the plate?



Experiment 3B

Aim Staining bacterial endospore

Key Terminology

- **Endospore** It is a dormant, tough and non-reproductive structure produced by gram-positive bacteria.
- **Sporulation** The process of a bacterium producing spores.
- **Metabolize** The process by which chemical changes in cells convert food into energy and assimilate nutrients.
- **Desiccation** Removal of moisture from the body (bacteria).

Basic Principle

Very few bacteria produce endospores, most notably the genera *Clestridium* and *Bacillus*. These protective structures are made through a process known as **sporulation** in response to extreme environmental conditions, such as high temperatures, desiccation, chemicals, change in pH and lack of food. In endospores in dormant state, bacteria do not **metabolize** or reproduce, but exist in a type of suspended animation, like plant seeds do. When environmental conditions again become favourable, the endospore germinates, returning the bacterium to its normal activity and reproducing metabolic state.

Normal water-based techniques, such as the gram-stain, will not stain these tough, resistant structures. In order to stain endospores, malachite green must be forced into the spore with heat, in much the same way that Carbol Fuschsin is forced through the waxy mycolic acid layer of *Mycobacterium* in the acid fast stain. Simple fluorescent technique is also available, developed by Schichmes et al. (2006) using acridine orange.

Materials

- Endospores of *Clestridium* and *Bacillus*
- Malachite green
- Safrinin stain
- Slides and cover slips
- Water bath
- Microscope (with oil immersion)

Methods

The protocol for differentially staining endospores and vegetative cells is as follows:

1. Place the heat-fixed bacterial slide over the screened water bath and then apply the primary stain Malachite green. If necessary, autoclave the slide for increasing the stainability.
2. Allow the slide to sit over the steaming water-bath for 5 min, reapplying the stain if it begins to dry out. Stainability differs with the time of heating, e.g. *Bacillus subtilis* var. *niger* endospores need 6 min.
3. Remove the slide from the water bath and rinse the slide with water until the water becomes clear.
4. Flood slide with the counter stain Safrinin for 20 seconds and then rinse. Use a cover slip to cover the area.
5. View the specimen under oil immersion (Magnification of 10×100 observations).

Observation

After the above staining procedure, the endospore will appear green, having retained the primary stain, Malachite green, the vegetative cells (bacteria in the active, metabolizing state) will appear pink, having retained the counter stain of Safrinin.

Figures

Draw the stained endospores and colour which you have observed under the microscope.

Precautions

- It should be noted that any debris on the slide can also take up and hold green stain creating confusion with endospores. Endospores are small and typically oval shaped. Large or irregular globes of green on the slide may be artifacts.

Viva Questions

- Why do we autoclave while staining an endospore?
- Under which conditions can endospore be germinated?
- Endospores are protective sexual or vegetative cells?
- Why does sporulation occur in bacteria?
- Why do we use safrinin while staining?

**Experiment 4**

Aim Determination of bacterial growth curve by

- (a) Manual counting method (by microscope), and
- (b) UV-spectrophotometer method.

Key Terminology

- **Manual counting** Score or count the number of bacterial cell per unit area.
- **Optical Density (O.D.)** Is the absorbance of an optical element for a given wavelength per unit distance.
- **Broth culture** A liquid nutrient medium for the culture of bacteria.
- **Lag-phase** Initial stage when bacteria are characterized by the active cell metabolism as a pre-requisite for cell division.
- **Log-phase** Exponential multiplication of bacteria after lag-phase.
- **Stationary-phase** Characteristic of bacterial multiplication after log-phase, when it slows down or stops cell division.
- **Death-phase** Last stage of bacterial multiplication, when the bacterial cells start dying.

Basic Principle

During the bacterial growth, they divide and multiply in number exponentially. After incubation,

- Acid-fast cells of *Mycobacterium* and *Nocardia* have waxy molecules in their cell walls that will take up and retain Malachite green stain when subjected to the endospore staining process. The uniformly green appearance of endospore stain acid-fast cells does not mean that they produce endospores. These are vegetative cells that have taken up colour from the heat, driving Malachite green into their waxy cell wall.

growth (i.e. development of many cells from a few cells) may be observed as one or a combination of three forms:

- (a) **Pellicle** A mass of organisms floating on top of the broth.
- (b) **Turbidity** The organisms appear as a general cloudiness throughout the broth.
- (c) **Sediment** A mass of organisms appears as a deposit at the bottom of the tube.

By assessing the growing bacteria at equal intervals of time at an optimum temperature can give their increase in number which may be plotted. The number of bacteria may be estimated by two ways:

(i) Manual Counting (By Microscope) Manually counting the number of bacterial cell per unit area, using microscope after regular interval.

(ii) UV-Spectrophotometer Measuring the Optical Density (O.D.) of broth culture at regular intervals of time by a UV Spectrophotometer. At ~600 nm wavelength, considering one O.D. unit corresponds to about 0.8×10^9 cells per ml.

In the initial stage (termed as **lag phase**) bacteria are characterized by the active cell metabolism, which is a pre-requisite for cell division. This is followed by exponential multiplication (or called **log phase**), i.e. characterized by active cell division. The next phase is known as the **stationary phase**, which has a characteristic slow down or ultimately stop cell division and is known as the constant stage and the last phase is the **death phase** (Fig. 3.4.1).

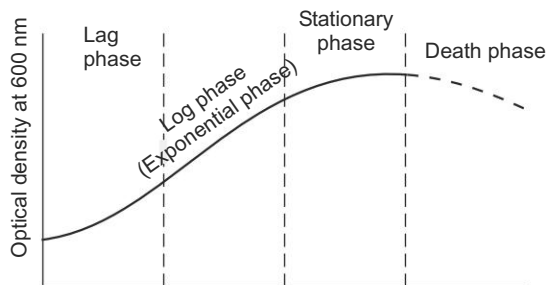


Fig. 3.4.1 A typical Sigmoid-growth curve in bacteria

Materials

- Inoculum of *E. coli* in slant (*Annexure III, No. 9*)
- Sterilized nutrient broth in- (a) 100 ml in 250 ml Conical flask, (b) 5 ml in culture tube
- Inoculating needle
- Sterilized 1 ml pipette
- Microscopic slides and cover slips
- Nutrient broth,
- Incubator set at 37°C
- Microscope
- UV- Spectrophotometer

- Shaker or Incubator shaker

Methods

1. Take a loopful of *E. coli* culture from the old slant using an inoculating needle and inoculate in 5 ml of nutrient broth in a tube. Incubate the tube at 37°C for 18–24 h.
2. Using a pipette inoculate 100 ml of nutrient broth in the conical flask with 1 ml of overnight *E. coli* culture.
3. Shake or swirl the content thoroughly and immediately withdraw 1 ml of broth. Place one drop of broth on a slide, spread uniformly using a needle and count the number of bacteria per unit area of microscopic field. Take an average of 10–15 observations.
4. In case the concentration of bacteria is very high, apply dilution method as explained in Experiment 1; (Methods, Steps 1 to 3). Back calculate the number of bacteria in the original broth and record.
5. Fill 2/3 of the cuvette with uninoculated broth and record OD at 600 nm in a spectrophotometer. This will represent as control or zero bacteria at zero time.
6. Check the OD of the broth immediately after inoculation.
7. Incubate the flask at 37°C on a shaker with ~200 rpm.
8. Withdraw 1 ml of broth at a regular interval of 1 h and repeat Steps 3 and 6. Continue the process for 20–24 h.

Data Tabulation

Tabulate your observation separately for bacterial frequency, Observe by manual counting using a microscope and OD by a spectrophotometer.

Time (in h)	No. of bacteria per unit area of microscopic field	OD* ₆₆₀	No. of bacteria
0			
1			
2			
⋮			
24			

* From above observation convert the OD to number of bacterial cells and record in the corresponding column using the conversion factor, i.e. one OD unit = 0.8×10^9 cells per ml.

Figure

Use a semi-logarithmic graph paper for plotting the number of bacteria per unit area of microscopic field or OD (or frequency of bacteria) along the Y-axis (logarithmic scale) against time along the X-axis (linear scale).

Results and Conclusions

- (a) *Microscope* Frequency of bacterial cell increases with time.
- (b) *UV-Spectrophotometer* OD of bacterial cell suspension increases with time.

- (c) Graph revealed four distinct phases of bacterial growth curves both using a microscope and a UV-spectrophotometer.

Precautions

- All tubes containing broth culture should be placed in a plastic beaker to keep them upright.
- Incubate the petri plates upside down (lid on the bottom) to prevent water from falling down on the growing colonies and causing them to run.
- Culture not more than 24 h old should be used as inoculum.

Viva Questions

- What is the pattern of bacterial growth curve we see in bacteria?
- What different phases do we observe in the bacterial growth curve?
- What is turbidity?
- Why do nutrient broths become turbid?
- In the bacterial growth curve, why do we see only four phases?

**Experiment 5****Aim**

Identification of bacteria through Biochemical test and Pigmentations.

Key Terminology

- **Biochemical testing** Chemical tests carried out for identification of bacteria.
- **Biochemical reaction** More specific test than biochemical test, so that bacteria may be identified to genus and species level.
- **Endo-enzyme** Enzymes which are situated inside or secreted by the bacterial cell.
- **Exo-enzyme** Contrary to endo-enzyme.
- **Hydrolyse (or Breakdown)** Breakdown (a compound) by chemical reaction with water.
- **Protein hydrolysis** Breaking down of peptide (short chain of amino acids) bonds of amino acids linked together.
- **Fermentation of carbohydrate** The chemical breakdown of a carbohydrate (substrate that serves as energy source) by bacteria, yeasts, or other microorganisms, producing acid or acid and gas.

- **Anaerobic Bacteria** requiring the absence of oxygen to bring about fermentation.
- **Aerobic** Contrary to anaerobic. Using or involving oxygen from the air for fermentation.
- **Catalase activity** Cause or accelerate a chemical reaction by acting as a substance without itself undergoing any permanent chemical change.
- **Chromogenic** Some microorganisms that produce pigments during growth.

Basic Principles

Biochemical Test Staining provides valuable information as to bacterial morphology, gram reaction, and presence of such structures as capsules and endospores. Beyond that, however, microscopic observation gives little additional information as to the genus and species of a particular bacterium. To identify bacteria, we

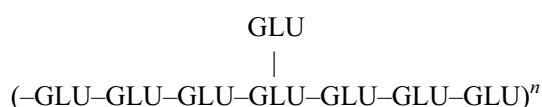
must rely on biochemical testing. The types of biochemical reaction each organism undergoes is very specific for its identification. This is based on the following reasons:

- Each different species of bacterium has a different molecule of DNA (i.e. DNA with a unique series of nucleotide bases).
- Since DNA codes for protein synthesis, the different species of bacteria even, by way of their unique DNA, will synthesise different enzymes.
- Enzymes catalyze all the various chemical reactions of which the organism is capable of. Different species of bacteria carry out different and unique sets of biochemical reactions.

When identifying a suspected organism, you use a series of differential media. After incubation, you may observe each medium to see if specific end products of metabolism are present. This can be done by adding indicators to the medium that react specially with the end product being tested, giving some form of visible reaction such as a colour change. The results of these tests on the suspected microorganism are then compared to known results for that organism to confirm its identification.

In general, we can classify enzymes as being either **exoenzymes** or **endoenzymes**. Exoenzymes are secreted by bacteria into the surrounding environment in order to break down nutrient molecules so they may enter the bacterium. Once inside the organism, some of the nutrients are further broken down to yield energy for driving various cellular functions, while others are used to form building blocks for the synthesis of cellular components. These latter reactions are catalyzed by endoenzymes located within the bacterium.

(i) Starch Hydrolysis Starch is a polysaccharide which appears as a branched polymer of the simple sugar glucose. This means that starch is really a series of glucose molecules linked together to form a long chain. Additional glucose molecules then branch off of this chain as shown below:



Some bacteria are capable of using starch as a source of carbohydrate but in order to do this, they must first **hydrolyse or break down** the starch into glucose so that it may enter the cell. The bacterium secretes an exoenzyme diastase which hydrolyses the starch to α -D-glucose by breaking the bonds between the glucose molecules.



Action of diastase

The glucose can then enter the bacterium and be used for metabolism.

(ii) Protein Hydrolysis Proteins are made up of various amino acids linked together in long chains by means of peptide bonds. Many bacteria can hydrolyse a variety of proteins into peptides (short chain of amino acids) and eventually into individual amino acids. They can then use these amino acids to synthesise their own proteins and other cellular molecules or to obtain energy. The hydrolysis of protein is termed **proteolysis** and the enzyme involved is called a **protease**. In this experiment, we will test for bacterial hydrolysis of the protein **casein**, the protein that gives milk its white, opaque appearance.

(iii) Fermentation of Carbohydrates Carbohydrates are complex chemical substrate which serve as energy source when broken down by bacteria and other cells. They are composed of carbon, hydrogen and oxygen (with hydrogen and oxygen being in the same ratio as water; $[\text{CH}_2\text{O}]$) and are classed as either sugar or starch.

Facultative anaerobic and anaerobic bacteria are capable of fermentation, an anaerobic process during which carbohydrates are broken down for energy production. A wide variety of carbohydrates may be fermented by various bacteria in order to obtain energy and the types of carbohydrates which are fermented by a specific organism can serve as a diagnostic tool.

We can detect whether a specific carbohydrate is fermented by looking for common end products of fermentation. When carbohydrates are fermented as a result of bacterial enzymes, the following fermentation end products may be produced:

(a) Acid end product, or (b) Acid and gas end products.

In order to test for these fermentation products, you inoculate and incubate tubes of media containing a single carbohydrate (e.g. lactose or maltose, a pH indicator Phenol red) and **Durham tube** (a small inverted tube to detect gas production). **If the particular carbohydrate is fermented by the bacterium, and end products are produced which lowers the pH, causing the pH indicator to change colour (e.g. Phenol red turns yellow).** If gas is produced along with the acid, it collects in the Durham tube as a gas bubble. **If the carbohydrate is not fermented, no acid or gas will be produced and the phenol red will remain red.**

Test for indole and hydrogen sulphide production of *Proteas mirabilis*, *E. coli* and *Enterobacter cloacae*, as well as, catalase activity of *Staphylococcus aureus* and *Streptococcus lactis* may also be studied, but these will not be included in this experiment. Instead, some special diagnostic tools (viz. colony pigmentation) for the identification of the organisms have been included for simplification.

Probably the most visual characteristic is **pigmentation** (colour). Some microorganisms produce pigment during growth and are said to be **chromogenic**. Often, however, formation of pigment depends on environmental factors such as temperature, nutrients, pH and moisture, e.g. *Serratia marcescens* produces a deep red pigment at 25°C, but does not produce pigment at 37°C.

Pigment can be divided into basic types: **water-insoluble** and **water-soluble**. If the pigment is water-insoluble, as in the case of most chromogenic bacteria, it does not diffuse out of the organism. As a result, the colonies are pigmented but the agar remains the normal colour. If the pigment is water-soluble as in the case of *Pseudomonas aeruginosa*, it will diffuse out of the organism into the surrounding medium. Both the colonies and the agar will appear pigmented. Below is a list of a few common chromogenic bacteria:

- *Staphylococcus aureus*—gold; water-insoluble
- *Micrococcus luteus*—yellow; water-insoluble
- *Micrococcus roseus*—pink; water-insoluble

- *Mycobacterium phlei*—orange; water-insoluble
- *Serratia marcescens*—orange/red; water-insoluble
- *Pseudomonas aeruginosa*—green/blue; water-soluble

Materials

- Starch agar (one plate) (See Annexure III, No.2)
- Skim milk agar (one plate) (See Annexure III, No.3)
- Phenol red lactose broth—3 tubes (See Annexure III, No.5)
- Phenol red maltose broth—3 tubes (See Annexure III, No.5)
- Tryptic soy broth culture of *Bacillus subtilis*, *E. coli* and *Staphylococcus aureus* (See Annexure III, No. 1)
- Iodine solution
- Phenol red
- Durham tube
- Inoculating needle
- Wax marking pencil
- Incubator set at 37°C.

Methods

Methods to conduct biochemical tests for these compounds will be described separately.

(i) Starch Hydrolysis

1. Using a wax marker, draw a line on the bottom of a starch agar plate so as to divide the plate in half. Label one half *B. subtilis* and the other half *E. coli*.
2. Make a single streak line with the appropriate organism on the corresponding half of the plate.
3. Incubate at 37°C until the next lab period.
4. In the next lab period, add iodine to see if the starch remains in the agar or has been hydrolyzed by the exoenzyme diastase. Iodine reacts with starch to produce a dark brown or blue/black colour. If starch has been hydrolysed there will be a clear zone around the bacterial growth, because the starch is no

longer in the agar to react with the iodine. If starch has not been hydrolysed, the agar will remain a dark brown or blue/black colour.

(ii) Protein Hydrolysis

1. Divide the skim milk agar plate in half and inoculate one half with *Bacillus subtilis* and the other half with *E. coli*, as was done in the above for starch agar plate.
2. Incubate at 37°C until the next lab period. If casein is hydrolysed, there will be a clear zone around the bacterial growth. If casein is not hydrolysed, the agar will remain white and opaque.

(iii) Fermentation of Carbohydrate

1. Label each tube with the name of the sugar in the tube and the name of the bacterium you are growing.
2. Inoculate one phenol red lactose broth tube and one phenol red maltose broth tube with *Bacillus subtilis*.
3. Inoculate a second phenol red lactose broth tube and a second phenol red maltose broth tube with *E. coli*.
4. Inoculate a third phenol red lactose broth tube and a third phenol red maltose broth tube with *Staphylococcus aureus*.
5. Incubate all tubes at 37°C until the next lab period.

Results

(i) Starch Hydrolysis

When iodine is added to starch, the iodine-starch complex that forms gives a characteristic dark brown or deep purple colour reaction. If the starch has been hydrolysed into glucose molecules by the diastase exoenzyme, it no longer gives this reaction.

Now flood the surface of the starch agar plate with gram's iodine.

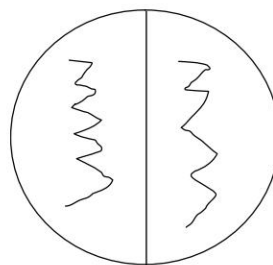
- If the bacterium produced an exoenzyme that hydrolyses the starch in the agar, a clear zone will surround the bacterial growth because the starch is no longer there to react with the iodine.

- If the bacterium lacks the exoenzyme to break down the starch, the agar around the growth should turn dark brown or blue/black colour due to the iodine starch complex.

(ii) Protein Hydrolysis

The protein casein exists as a colloidal suspension in milk and gives its characteristic white, opaque appearance. If the casein in the milk is hydrolysed into peptides and amino acids, it will lose its opaqueness.

- If the bacterium produced an exoenzyme capable of hydrolysing the casein, there will be a clear zone around the bacterial growth.
- If the bacterium lacks the exogenous to break down casein, the skim milk agar will remain white and opaque.
- Record your results and indicate which organism was capable of hydrolysing the starch (+ = hydrolysis; – = no hydrolysis).



Escherichia coli

Bacillus subtilis

Casein hydrolysis = Casein hydrolysis =

(iii) Fermentation of Carbohydrates

As mentioned above, we can detect whether a specific carbohydrate is fermented by looking for common end products of fermentation. When carbohydrates are fermented as a result of bacterial enzymes, there will be either (a) Acid or (b) Acid and gas. The results of fermentation may be acid alone or acid plus gas, but never gas alone.

Phenol red pH indicator appears red or orange at neutral pH and appears yellow or clear at an acid pH.

- A change in colour in the tube from red or orange to yellow or clear indicates that the organism has fermented that particular carbohydrate, producing acid end products.

- A substantial gas bubble at the top of the Durham tube, the inverted test tube within the broth, indicates gas was also produced from the fermentation of the carbohydrate.
- If the phenol red remains red, no acid was produced and the carbohydrate was not fermented.

Following are three alternative possible results:

- Carbohydrate fermentation producing **acid**

but no gas : acidic (yellow or clear); no substantial gas bubble in the Durham tube.

- Carbohydrate fermentation producing **acid and gas**: acidic (yellow or clear); a substantial gas bubble in the Durham tube.
- No carbohydrate fermentation. No acid or gas (neutral pH—red or orange); no substantial gas bubble in the Durham tube.

Organism	Phenol Red Maltose	Phenol Red Lactose
<i>Bacillus subtilis</i>		
Acid		
Gas		
Fermentation		
<i>E. coli</i>		
Acid		
Gas		
Fermentation		
<i>Staphylococcus aureus</i>		
Acid		
Gas		
Fermentation		

Record your results + = positive; – = negative

Precautions

- Carefully check the colour development in different tests.
- Label the plates and tubes properly.

Viva Questions



- How do you test the presence of sugar?
- Describe the method of testing for casein hydrolysis.
- Name the general end product which may be formed as a result of bacterial fermentation of sugar.
- How end product of bacterial fermentation of sugar changes the appearance of a tube containing sugar, the pH indicator phenol red and a Durham tube?
- Give example of a chromogenic bacterium.



Experiment 6

Aim

Uses of physical and antimicrobial agents to control microorganisms.

Key Terminology

- **Physical agents** A substance that performs the action of controlling microorganism.
- **Chemical agents** Chemical substances that act as an agent to control microorganisms.

- **Hypotonic** Containing high concentration of essential salts and minerals in the cell, so that water flows into the cell, making the cell walls of bacteria and fungi rigid.
 - **Isotonic** Concentration as in the cell. Water flows equally in and out of the cell.
 - **Hypertonic** Contrary to hypotonic. Solution or salt and mineral concentration outside the cell is higher, so that water flows out, resulting in shrinking of the cytoplasmic membrane.
 - **Filtration** Move gradually through microbiological membrane which filters (or screen) and provide sterilization of materials.
 - **Antimicrobial** Any substance, chemical or agent that is harmful to microorganisms.
 - **Chemotherapeutic agent** The treatment with substances or chemicals that inhibits or kills the microorganisms.
 - **Antibiotic** A chemically synthesized substance or medicine that inhibits the growth of or destroys bacteria.
2. **Disinfection** Elimination of microorganisms from inanimate objects or surface.
 3. **Decontamination** Treatment of an object or inanimate surface to make it safe to handle.
 4. **Disinfectant** An agent used to disinfect inanimate objects but generally too toxic to use on human tissues.
 5. **Sanitizer** An agent that reduces, but may not eliminate, microbial number to a safe level.
 6. **Antiseptic** An agent that kills or inhibits growth of microbes but is safe to use on human tissue.
 7. **Antibiotic** A metabolic product produced by one microorganism that inhibit or kills other microorganisms.
 8. **Chemotherapeutic antimicrobial chemical** Synthetic chemicals that can be used therapeutically.
 9. **Cidal** (act of killing) An agent that is cidal in action will kill microorganisms and viruses.
 10. **Static** (lacking movement) An agent that makes the microorganisms static in action and will inhibit the growth of microorganisms.

Basic Principle

This experiment will deal with the inhibition, destruction and removal of microorganisms. Control of microorganisms is essential to prevent the transmission of diseases and infection, stop decomposition and spoilage and prevent unwanted microbial contamination. Microorganisms are controlled by means of (a) **Physical agents**, and (b) **Chemical agents**. Physical agents include such methods of control as high or low temperatures, desiccation, osmotic pressure, radiation and filtration. Control by chemical agents may be further sub-divided into (i) disinfectants, antiseptic and sanitizers, and (ii) antimicrobial chemotherapeutic agent's, viz. antibiotic susceptibility, etc.

Basic common terms used in discussing the control of microorganisms include:

1. **Sterilization** Destroying all living organisms and viruses. A sterile object is one free of all life including bacterial endospores, as well as viruses.

In this experiment, we will deal with the control of microorganisms with physical agents and antimicrobial chemotherapeutic agent's, viz. antibiotics.

A. Physical Agents

Following are some of the agents usually used for control of microorganisms:

1. Temperature

Temperature is a very common and effective way of controlling microorganisms. Microorganisms have a **minimum**, an **optimum**, and a **maximum** temperature for growth. Temperature below the minimum usually has a **static action** on microorganisms. They inhibit microbial growth by slowing down but do not necessarily kill the organism. Temperature above maximum usually has a **cidal action**, as they denature microbial enzymes and other proteins. (Also see Chapter I, Page 27)

(a) High Temperature

Vegetative microorganisms can generally be killed at temperature from 50° to 70°C with moist heat. Bacterial endospores, however, are very resistant to heat and extended exposure to much higher temperature is necessary for their destruction. High temperature may be applied as either moist or dry heat.

(i) Moist heat Moist heat is generally more effective than dry heat for killing microorganisms because of its ability to penetrate microbial cells. Moist heat kills microorganisms by denaturing their proteins (causes proteins and enzymes to lose their three-dimensional functional shape). It also melts lipids in cytoplasmic membranes.

(a) Autoclaving: Autoclaving employs steam under pressure. Water boils at 100°C, however, when put under pressure, water boils at a higher temperature. During autoclaving, the materials to be sterilized are placed under 15 pounds per square inch of pressure in a pressure-cooker type of apparatus. When placed under 15 pounds of pressure, the boiling point of water is raised to 121°C, a temperature sufficient to kill bacterial endospores. The time the material is left (about 15–45 min) in the autoclave varies with the nature and amount of material being sterilized. It has a cidal effect both for vegetative organisms and endospores (see Chapter 1, Page 27).

(b) Boiling water: Boiling water (at 100°C) will kill vegetative cells after 10 min of exposure. However, certain viruses (e.g. Hepatitis viruses) may withstand for 30 min and endospores of *Clostridium* and *Bacillus* species may survive even hours of boiling.

(ii) Dry heat It kills microorganisms through a process of protein oxidation rather than protein coagulation.

(a) Hot air sterilization: This process is used generally for sterilizing glasswares, metallic instruments and other inert materials, e.g. oils and powders which are not damaged by high temperature. Microbiological ovens employ very high dry temperatures, e.g. 171°C for 1 h; 160°C for 2 h or longer or 121°C for 16 h or longer depending on the volume.

(b) Incineration: It is used to destroy disposal or expendable material by burning, e.g. inoculating loops.

(c) Pasteurization: Mild heating of milk and other materials to kill particular spoilage organisms or pathogens. Milk is pasteurized by heating to 71.6°C at least 15 sec in the flash method or 62.9°C for 30 min in the holding method. However, it does not kill all organisms.

(b) Low Temperature

Inhibits microbial growth by slowing down microbial metabolism, e.g. refrigeration and freezing. Refrigeration at 5°C slows the growth of microorganisms and keeps food fresh for a few days. Freezing at –10°C can stop microbial growth without killing and keeps food fresh for several months.

2. Desiccation

Desiccation or drying generally has a static effect on microorganisms. Lack of water inhibits the action of microbial enzymes. Dehydrated and freeze-dried foods.

3. Osmotic Pressure

Microorganisms, in their environments, are constantly faced with alteration in osmotic pressure. Water tends to flow through semi-permeable membranes, e.g. the cytoplasmic membrane of microorganisms, towards the side with a higher concentration of dissolved material (solute) or water will move from greater water (lower solute) concentration to lesser water (greater solute) concentration. There can be three different types of environmental situations.

(i) Hypotonic Environment Water will flow into the cell. The rigid cell walls of bacteria and fungi, prevent bursting or **plasmolysis**.

(ii) Isotonic Environment Water flows equally in and out of the cell.

(iii) Hypertonic Environment A situation is created when solute is higher outside of the cell than inside. Under this condition, **water flows out of the cell**, resulting in shrinking of the cytoplasmic membrane or plasmolysis. The cell becomes

dehydrated and its growth is inhibited, e.g. canning of jams or preservation with a high concentration or salt-curing meats or placing foods in a salt-brine have static action of and prevent bacterial decomposition.

4. Radiation

There are generally two type of radiations, viz. (a) **Non-ionizing**, e.g. Ultraviolet (UV) radiation and (b) **Ionizing**, e.g. X-rays, gamma rays, etc.

(a) **Ultraviolet Radiation** The ultraviolet portion of the light spectrum includes all radiations with wavelengths from 100 to 400 nm. It has a low wavelength and low energy. The microbial activity of UV light depends on the **length of exposure**, the longer the exposure the greater will be the cidal activity. It also depends on the wavelength of UV used. The most cidal wavelength of UV light lies in the 260 to 270 nm range, where it is absorbed by nucleic acid. However, it has very poor penetrating power.

(ii) **Ionizing Radiation** Has much more energy and penetrating power than UV radiation. It ionizes water and other molecules to form radicals that can disrupt DNA molecules and proteins. It is often used to sterilize pharmaceuticals and disposable medical supplies such as syringes, surgical gloves, catheters and petri-plates. It can also be used to retard spoilage in sea-foods, meats, poultry and fruits.

5. Filtration

Microbiological membrane filters provide a useful way of sterilizing materials, e.g. vaccines, antibiotic solutions, animal sera, enzyme solutions, vitamin solution and other solutions that may be damaged or denatured by high temperatures or chemical agents. The filters contain pores small enough to prevent the passage of microbes but large enough to allow the organism-free liquid to pass through. The liquid is then collected in a sterile flask. Filters with a pore diameter from 25 to 450 nm are usually used in this procedure. Filters can also be used to remove microorganisms from water and air for microbiological testing.

B. Chemical Agents

As mentioned earlier, chemical agents may be further subdivided into

(i) **Disinfectants, antiseptics and sanitizers (which will not be discussed).**

(ii) **Antimicrobial chemotherapeutic agents**

Based on their origin, there are two general classes of chemical agents. For example:

(a) **Antibiotic** Substances produced as metabolic products of one microorganism which inhibit or kill other microorganisms **(will be discussed).**

(b) **Antimicrobial chemotherapeutic chemicals** Chemicals synthesized in the laboratory which can be used therapeutically on microorganisms **(which will not be discussed).**

Antibiotic Since nowadays many antibiotics are extensively modified in the laboratory (semi-synthetic) or even synthesized without the help of microorganisms, it is difficult to draw distinction between the two classes.

Most of the major groups of antibiotics were discovered prior to 1955, and most antibiotic advances since then have come about by modifying the older forms. In fact, only three major groups of microorganisms have yielded useful antibiotics. The actinomycetes (filamentous, branching soil bacteria, e.g. *Streptomyces*), bacteria of the genus *Bacillus* and the saprophytic moulds *Penicillium* and *Cephalosporium*.

Some antimicrobial agents are cidal in action; they **kill microorganisms**, e.g. penicillins, cephalosporins, streptomycin, neomycin. Others are static in action: they **inhibit microbial growth** long enough for the body's own defences to remove the organisms, e.g. tetracyclines, erythromycin, sulfonamides. These agents may also vary in their spectrum. Drugs that are effective against a variety of both gram-positive and gram-negative bacteria are said to be **broad-spectrum**, e.g. tetracycline, streptomycin, cephalosporins, ampicillin, sulfonamides. Those effective against just gram-positive bacteria, just gram-negative bacteria, or only a few species are termed **narrow-spectrum**, e.g. penicillin G, erythromycin, clindamycin, gentamycin. In fact, indiscriminate use of broad-spectrum antibiotics should be avoided, as it leads to superinfection by opportunistic microorganisms, e.g. *Candida* (yeast infection). Indiscriminate uses of antimicrobial chemotherapeutic agents include

drug toxicity, allergic reactions to the drug and selection for resistant strains of microorganisms. At present fourth generation antibiotic drugs are in use and selection of and availability of appropriate drug becomes difficult. As a result, continuous and indiscriminate use of antimicrobial chemotherapy leads to the development of **resistant strains** of bacteria. There are various mechanisms by which most of the bacteria become resistant to antimicrobial agents.

Materials

For Physical Agents

(a) Effect of Osmotic Pressure

- Tryptic soy broth culture of *E. coli* and *Staphylococcus aureus* and spore suspension of the mould *Aspergillus niger* (See Annexure III, No.1)
- One petri-plate each of following tryptic soy agar:
 - Tryptic soy agar (control)
 - Tryptic soy agar with 5% Glucose
 - Tryptic soy agar with 10% Glucose
 - Tryptic soy agar with 25% Glucose
 - Tryptic soy agar with 5% NaCl
 - Tryptic soy agar with 10% NaCl
 - Tryptic soy agar with 15% NaCl
- Inoculating needle,
- Glass marking pencil,
- Incubator set at 37°C.

(b) Effect of UV Radiation

- UV radiation source
- Tryptic soy agar—5 plates with *S. marcescens*
- Glass spreader or sterile swab
- Glass marking pencil

(c) Effect of Filtration

- Tryptic soy agar plates—two
- Membrane filter
- Filtration device
- Forceps and spirit lamp

For Chemical Agents

(d) Testing microbial resistance and susceptibility to antibiotics by Bauer-Kirby disc diffusion test:

- ∞ Test bacterium grown on tryptic soy agar petri-plates,
- ∞ Different concentrations of standard antibiotic containing discs
- ∞ Inoculum of *Pseudomonas*, *S. aureus* and gram-negative enteric bacilli *E. coli*, *Proteus*, etc.

Methods

For Physical Agents

(a) Test for Autoclaving

1. Take four culture tubes containing tryptic soy agar.
2. Autoclave two tubes and leave the other two tubes as such.
3. Incubate all the four tubes together at room temperature until the next lab period.
4. Differentiate between two autoclaved and two non-autoclaved tubes. Record your observation.

(b) Test for Osmotic Pressure

1. On the lower plate divide each plate into three equal parts using a glass marking pencil.
2. Using inoculating loop, streak one part of each plate with *E. coli*, the second part with *S. aureus* and the third part with *A. niger*.
3. Incubate at 37°C until the next lab period. Record your observations.

(c) Test for UV Radiation

1. Streak all five plates of tryptic soy agar with *S. marcescens* using glass spreader or sterile swab (so as to cover the entire agar surface with organisms).
2. Expose **three** of the plates to UV light as follows:
 - (a) Remove the lid of each plate and place a piece of cardboard with the letter “V” cut out of it, over the top of the agar.
 - (b) Expose the first plate to UV light for **1 sec**, the second plate for **3 sec** and the third plate for **10 sec**.
 - (c) Replace the lids and incubate at room temperature until the next lab period.
3. **Leaving the lid on**, lay the card-board with the letter “V” cut over the fourth plate and

expose to UV light for 30 sec. Incubate at room temperature with the other plates.

4. Use the fifth plate as a non-irradiated control and incubate at room temperature with the other plates.
5. Record your observation together in the next lab period.

(d) Test for Filtration

1. Take two plates of tryptic soy agar.
2. Using alcohol-flamed forceps, aseptically place a sterile membrane filter into a sterile filtration device.
3. Pour the culture (of any microorganism) into the top of the filter set-up.
4. Vacuum until all the liquid passes through the filter into the sterile flask.
5. With alcohol-flamed forceps, remove the filter and place it with organism-side up on the surface of a tryptic soy agar plate
6. Using a sterile swab, streak the surface of another tryptic soy agar plate with filtrate from the flask.
7. Incubate the plates at 37°C until the next lab period.
8. Record your observation together in the next lab period.

(e) For Antibiotic Susceptibility Test by Bauer-Kirby Test To determine antimicrobial susceptibility using the Bauer-Kirby disc diffusion method *in vitro*, response of bacteria to a standard antibiotic-containing disc.

1. Inoculate the test bacteria on tryptic soy agar plate, e.g. *Pseudomonas*, *S. aureus*, and *E. coli* (gram-negative bacterium), so as to cover the entire agar surface uniformly (spread the entire plate with the help of a

glass spreader or cotton swab). Swab the entire plate from top to bottom, edge-to-edge, leaving no gaps.

2. Place standard antibiotic-containing discs on the plates inoculated with different bacteria (use same or different concentration of antibiotic-containing discs).
3. Incubate the plate agar side up at 37°C for 16–18 h.
4. Measure the diameter of any resulting **zones of inhibition** (clear zone seen on plate) in millimetre (mm) using divider (Fig. 3.6.1).

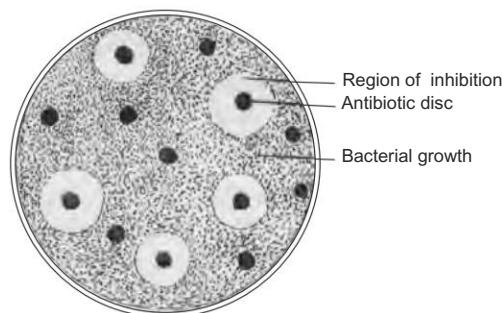


Fig. 3.6.1 Region of inhibition by antibiotic discs

5. Determine if the bacterium is **susceptible, moderately susceptible, intermediate** or **resistant** to different concentration of discs.
6. Record your observation together in the next lab period.

Observations

For Physical Agents

(a) Autoclaving Differentiate between the autoclaved and the non-autoclaved culture tubes. Record the differences in the following table:

	Description of the tubes
Autoclaved	
Un-autoclaved	

(b) **Osmotic Pressure** Observe the seven plates from the osmotic pressure experiment and record the results in the table given below:

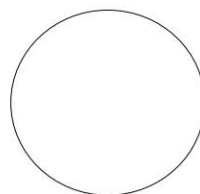
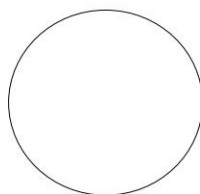
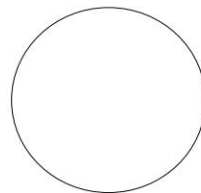
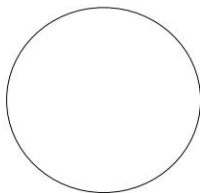
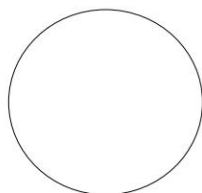
Plate	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Aspergillus niger</i>
Control			
5% Glucose			
10% Glucose			
25% Glucose			
5% NaCl			
10% NaCl			
15% NaCl			

(c) **UV radiation**

Non-irradiated control

1 sec UV exposure lid off

3 sec UV exposure lid off



10 sec UV exposure lid off

30 sec UV exposure lid on

(d) **Filtration** Observe the two filtration plates and describe the results below.

Plate containing the filter (Growth or no growth)	
Plate streaked with filtrate (Growth or no growth)	

(e) **Interpret the results and record your results in the following table:**

Antimicrobial agent	Zone (in mm)	R*	I*	MS*	S*
Amoxicillin/ Clavulanic acid					
Ciprofloxacin					
Erythromycin					
Kanamycin					

* R = Resistant; I = Intermediate; MS = Moderately susceptible; S = Susceptible

Precautions

- Do not look directly at the UV light as it may harm the eyes.
- While inoculating with cotton-swab, squeeze the swab against the inner wall of the tube to remove excess liquid.

Viva Questions

- Define the following terms: disinfection, decontamination, sterilization, static and cidal.
- Is moist or heat more effective in controlling microorganisms and indicate why?
- How does moist heat kill microorganisms?
- How does dry heat kill microorganisms?
- What is pasteurization?
- Low temperature has a static or cidal effect?
- Does desiccation has a static or cidal effect?
- What are the differences between hypotonic, hypertonic and isotonic?
- Differentiate between plasmolysis and plasmolysis.
- How is bacterial growth inhibited in jam and salt-cured meats?
- State whether hypertonic has a static or cidal effect on microorganisms.
- Does wavelength and the length of exposure influence the bacteriological effect of UV light?
- How does UV light kill microorganisms?
- Mention some application of ionizing radiation that kills microorganisms?
- How does the concept of micro-pore membrane filter work in sterilization of solutions?
- Why are filters preferred over autoclaving for vaccines, antibiotic solutions, sera and enzyme solutions?
- Why is antimicrobial susceptibility testing often essential?
- How can this method be used to isolate oil-degrading bacteria?

**Experiment 7**

Aim Determine which type of garbage is Bio-degradable and which are non-degradable.

Key Terminology

- **Bio-degradable** Substances (or wastes) that can be broken down or decomposed by bacteria or other living organisms.
- **Non-degradable** Contrary to bio-degradable. Substances that cannot be broken down or decomposed by living organisms.

Basic Principle

The disposal of solid waste materials has become a serious problem for many communities. Disposal of garbage from metros are a Herculean task. It requires both labour and cost for disposing them.

Generally, there are two types of garbage, viz. (i) **Bio-degradable**, and (ii) **Non-degradable**. The latter type may be further classified into building materials, metal, glass, aluminum foils, cans, etc.

(i) **Bio-degradable** garbage which can be or degraded by microorganisms, e.g. paper, vegetables, fruit pieces, meat, bones, etc.

(ii) **Non-degradable** microorganisms are unable to destruct or degrade, e.g. polythene, styro-foam (polystyrene + foam), thermocol, etc.

Some garbage, e.g. paper, vegetables, fruit pieces, etc. contain cellulose. There are many cellulose degrading bacteria and fungi present in the soil, viz. *Trichoderma reesci*, *T. viride*, *Clostridium*

thermocellum, *Streptomyces* sp., *Fusarium solani* and fungi *Aspergillus niger*, *A. fumigatus*, *A. acculeatus*. As such, any bacteria or fungi that synthesize or secrete enzyme cellulose exogenously will be useful in bio-degradation of garbage by breaking down the bonds between glucose molecules and convert cellulose to β -glucose.

A common way of disposing garbage in our country is filling up of pits or waste lands, which is subsequently covered with soil to create an anaerobic environment. It stimulates the condition and helps the bacteria or fungi to work affectively. But the amount of garbage which is being produced by the Metros, as well as in big cities is alarming and posing challenge to the scientists and environmentalists. Therefore, new techniques of disposal and degradation of wastes are necessary. However, while working out the methodology, one should take into account its impact on the environment and the cost-effectiveness of the technology.

Materials

- Petri-dishes-4 pairs
- Cellotape
- Garden soil
- Dropping bottle with sterilized water
- Sample of garbage containing, viz. paper, vegetables, fruit pieces, meat, bones, polythene, thermocol, etc.
- Wax marking pencil.

For Extension Experiments

- Plastic bags—5 Nos.
- Candle
- Bell Jars- 2 Nos.
- Cellotape
- Petri-dishes
- Weighing balance
- Nutrient agar,
- Acetone

Methods

1. Level the bottom of four petri-dishes from 1 to 4, write your name and the date on each dish. Divide the bottom of the dish into three equal sections, using 1 wax pencil.
2. Gather 12 small samples of garbage. Cut the samples into 2 sq. cm.
3. Put three samples in each petri-dish—one in each section.
4. Record the nature of the sample materials in each dish. Indicate the size, colour, texture, dour or any other features important. Predict how each of the sample will look after one week or more.
5. Cover the sample material with garden soil. Use a Dropping bottle to add enough sterile water to make the soil moist but not soggy.
6. Cellotape the lid on to each dish and keep at room temperature. Examine after one week or more.

This experiment may be extended to three of the following useful studies.

Extension I

Create an anaerobic environment by putting the petri-dishes with garbage samples inside a bell jar with a burning candle. Place a set of petri-dish containing garbage samples and soil inside the bell jar. Cellotape the jar, so that atmospheric air may not get in. Leave for a week. Compare the amount of degradation in an aerobic environment to the anaerobic environment.

Extension II

After one week, make water dilution out of partially degraded garbage and follow Experiment 1 (in this chapter). You may also find out the type of organisms and describe the nature of colony as well as the type of enzyme responsible for degradation (see in this Chapter, Experiment 5, Starch hydrolysis).

Extension III

For one full day, collect the garbage and trash from your house.

Divide this into five plastic bags each containing

Bag # 1: Glass (broken) and bottles

Bag # 2: Aluminium foils or cans

Bag # 3: Plastic containers, Polythene

Bag # 4: Paper goods

Bag # 5: Wet garbage

Weigh each bag using a balance, to find the mass of each bag. Using this data predict the quantity of garbage your family will produce in one day, one week and one year.

Observations and Results

After one week examine the dish and record any change in size, colour, odour, texture of the sample used in this experiment.

Sample used	Nature of garbage (At the beginning)	Nature of garbage (After one week)
Paper		
Size		
Colour		
Odour		
Texture		
Weight		

For Extension I

Garbage Sample	Nature of aerobic garbage	Nature of anaerobic garbage
Paper		
Food sample		
Aluminium foil		
Polythene		

For Extension III

Bag No.	Weight after one week (in g)	Weight after one month (in g)	Weight after one year (in g)
# 1			
# 2			
# 3			
# 4			
# 5			

Precautions

- Wear goggles and gloves while working with iodine and garbage.
- Use suitable forceps or tong or spoon for separating garbage.

Viva Questions

- What type of enzyme do bacteria synthesis for degrading starch?
- How could the method be used to isolate oil-degrading bacteria?
- Summarize this lab experience and include possible application of this procedure to help clean up the environment.
- Why are the plates containing starch effective in isolating starch degrading bacteria?
- Explain the possible application of this procedure to help clean up the environment



Experiment 8

Aim Isolation and Identification of Non-Pathogenic Fungi.

Key Terminology

- **Hyphae** Each of the branching filaments that make up the mycelium of a fungus.
- **Septa** A partition separating two cells or chambers.
- **Mycelium** A network of fine white filaments (hyphae) constituting the vegetative part of a fungus.
- **Chemo-heterotrophs** Fungus requiring organic compounds for their energy and nutrients by absorbing carbon source from their environment.
- **Saprophytes** A plant, fungus, or micro-organisms that live/s on decaying matter.
- **Parasite** An organism or plant which lives in or on another organism and get their nutrition for growth or living.
- **Mycotic infection** (or **mycoses**) Disease caused by fungi.
- **Pathogenic** A microorganism that can cause disease.
- **Mycotoxin** A harmful compound produced by *Aspergillus* by growing in certain foods.
- **Columella** Sporangiospores produced by *Rhizopus*, at the end of which appears a dome-shaped structure.

Basic Principle

Fungi are eukaryotic organisms which include yeasts, moulds and fleshy fungi. Yeasts are microscopic, unicellular; moulds are multi-nucleated, filamentous, composed of **hyphae** that are branched tubular structure from 2–10 μm in diameter and are usually divided into cell-like units by cross walls called **septa** (e.g. mildews, rusts and common household moulds). The total mass of hyphae is termed **mycelium**. The function of mycelium is to anchor the mould and absorb nutrients. The portion which produces asexual

reproductive spores is known as **aerial mycelium**. Fleshy fungi include mushrooms and puffballs.

All fungi are chemo-heterotrophs that require organic compounds for their energy and carbon source. They obtain these nutrients by absorbing them from their environment. Most of them live on decaying organic material and are termed **saprophytes**, whereas others are parasites, getting their nutrients from living plants and animals. Moulds reproduce primarily by means of asexual reproductive spores, e.g. conidiospores, sporangiospores and anthrospores. These spores are disseminated by air, water, animals. It may also reproduce by means of sexual spores, viz. ascospores and zygospores. The main criteria for identifying and classifying moulds, depends on the form and manner in which the spores are produced, appearance of the hyphae and mycelium.

The study of fungi is termed **mycology** and the diseases caused by fungi are called **mycotic infection** or **mycoses**. In general, **non-pathogenic** fungi are beneficial to human beings. They are involved in the decay of dead plants and animals, the manufacturing of various industrial and food products, production of many common antibiotics and may be eaten as food. However, some fungi are **pathogenic** which damage woods, fabrics, spoils foods and are responsible for causing a variety of plant and animal diseases including human infections. In this experiment we will concentrate our studies on non-pathogenic fungi, viz. moulds (e.g. *Penicillium*, *Aspergillus*, *Rhizopus*) and yeasts (e.g. *Saccharomyces cerevisiae*). With the help of microscope, we will study their structure, colony appearance, colour and type of asexual spores produced.

(i) Non-pathogenic Moulds

- (a) *Penicillium* is one of the most common household moulds and is a common food contaminant. The conidiospores of *Penicilli-*

um usually appear as grey, green or blue and are produced in chains on finger-like projections called **phialides**.

(b) *Aspergillus* is another non-pathogenic mould, grows in certain foods and can produce **mycotoxins**. The conidiophore terminates in a ball-like structure called a **vesicle**. Conidiospores are brown to black, produced in chains on phialides coming out of the vesicle.

(c) *Rhizopus* The sporangiospore are produced in a sac-like structure called **Sporangium** which has a dome-shaped **collumella**. Typical brown or black sporangiospores are produced within the sporangium. On the vegetative hyphae, anchoring structures called **rhizoids** are also produced.

Non-pathogenic moulds are commonly cultured on fungal-selective or enriched media, e.g. as Saboraud Dextrose agar (SDA), Corn Meal Agar (CMA) and Potato Dextrose agar (PDA).

(d) *Saccharomyces* are unicellular, oval or spherical fungi, which increase in number asexually by a process termed **budding**. Usually the bud breaks away to become a new daughter cell. Because of their unicellular and microscopic nature, yeasts colonies resemble bacterial colonies on solid media. Certain dimorphic fungi are able to grow as yeast or as a mould, depending on growth conditions. Yeasts are facultative anaerobes and can therefore, obtain energy by aerobic respiration and anaerobic fermentation. The large majority of yeasts are non-pathogenic and some are of great value in industrial fermentation, e.g. *Saccharomyces species* are used for baking and brewing.

Materials

- Four plates each of (a) Saboraud Dextrose agar (See Annexure III, No. 4)
- (b) Mycosel agar (See Annexure III, No. 7)
- (c) Corn meal agar (See Annexure III, No. 6)
- (d) Potato dextrose agar (See Annexure III, No.4)
- Microscope
- Slides and coverslips

- Alcohol
- Forceps
- Spirit lamp or burner
- Wax marking pencil
- Inoculating needle or sterile swabs

Methods

1. Using an inoculating needle streak all the four plates with *Penicillium*. Similarly, repeat it with *Aspergillus*, *Rhizopus* and *Saccharomyces cerevisiae*.
2. Pick up a glass cover slip with forceps, dip the cover slip in alcohol, and ignite with flame of lamp or burner and place it over a portion of the streak line so that the plates can be observed directly under the microscope after incubation. Repeat streaking for the 2nd, 3rd and 4th microbes.
3. Incubate at room temperature until the next lab period.
4. Remove the lids of the agar plates one by one and put the plates on the stage of the microscope. Observe an area under the cover slip that appear hazy to the naked eye.
5. Reduce the light by adjusting the iris diaphragm lever to extreme right. Raise the stage gradually using the coarse adjustment screw and then lower the stage keeping your eye on the eye piece until the mycelial growth or yeast cells comes into focus. Repeat all the plates one after another. Also prepare slides and observe under high magnification.

Observations

Observe the following:

1. Describe the asexual spores, e.g. conidiospores and sporangiospores and name a mould or yeast that is produced by each of these moulds and yeast.
2. State the main criteria used in identifying moulds and yeasts.
3. Note their colour and appearance on different media.
4. Make drawings of the moulds and yeast as they appear microscopically under high magnification.

Results

Write the description for different medium

Organism	Saboraud Dextrose agar	Mycosel agar	Potato Dextrose agar	Corn Meal agar
<i>Penicillium</i> *				
(a) Conidiospore				
(i) Colour				
(ii) Shape				
(iii) Colour of media				
(b) Sporangiospore				
(i) Colour				
(ii) Shape				
(iii) Colour on the media				

* Make similar tables for *Aspergillus*, *Rhizopus* and *Saccharomyces*

Figures

<i>Penicillium</i>	<i>Aspergillus</i>
<i>Rhizopus</i>	<i>Saccharomyces</i>

Precautions

- Before inoculation mark the plates carefully mentioning the media and moulds or yeast used for inoculation.
- Be careful during adjustment of microscope coarse adjustment knob. Objective should not touch the cover slips or slides.
- Do not crush or press hard after placing the cover slip.
- Adjust the diaphragm light carefully to study the colours of conidiospores or sporangiospores.

Viva Questions

- Define hypha, mycelium, conidiospore, sporangiospore, and mycosis.
- Mention some of the uses of fungi to human beings.
- What are the two ways the yeast *Saccharomyces* is beneficial to human?
- Name a common yeast that infects human.
- What are the criteria for recognizing *S. cerevisiae*?
- What are phialides?
- What is a vesicle?

**Experiment 9****Aim**

Determine the cell viability by dye-exclusion method.

Key Terminology

- **Viability** Capable of surviving successfully.
- **Cyclosis** Cytoplasmic streaming observed under a phase contrast microscope.
- **Permeable** Capable of allowing liquids or gases to pass through.
- **Plasma membrane** Cytoplasm or protoplasm which behaves like a filter.

Basic Principle

For raising microbial, plant or animal cell cultures a high percentage of viable cells are required. A common stain **Fluorescent diacetate** (FDA) or **Evan's blue**, is most routinely used for determining cell viability. There are several techniques to test cell viability. Some of them are as follows:

1. Use of phase contrast microscope to see cytoplasmic streaming (known as **Cyclosis**), which is an indication of active cell metabolism
2. Measuring O₂ uptake (using oxygen electrode) to check respiratory metabolism
3. In case of plants photosynthesis activity is measured
4. The presence of healthy nucleus.

In this experiment we will make use of Evan's blue which works just complementary to FDA dye. FDA is an important non-fluorescent and non-polar compound, and is **readily permeable across the plasma-membrane**. This compound is broken inside the cell by an enzyme esterase (by hydrolysis) into a fluorescent polar portion which is **not permeable through plasma-membrane**. Thus, it accumulates in intact cell cytoplasm but not in broken or dead cells (where it gets lost). Therefore, only the **intact cells show green fluorescence** due to FDA treatment, and they are considered as viable cells when Evan's is used. Only dead or damaged cells take up the stain (appear as blue cells), and viable and intact cells remain unstained. The percentage of viable cells (non-

stained) can be calculated out of the total number of cells by counting with the help of a microscope.

Percent viability is calculated by applying the formula:

$$\% \text{viability} = \frac{\text{No. of Non-blue Cells}}{\text{Total No. of Cells}} \times 100$$

Materials

- ∞ Microscope
- ∞ Microscopic slide and cover slips
- ∞ 0.1% Evan's blue stain
- ∞ Freshly dehisced pollen from flowering plants or cultured cells of plants or animals
- ∞ Mannitol or Sorbitol

Methods

1. Prepare 0.1% Evan's blue in 0.4M mannitol or sorbitol.
2. Small fraction of pollen or cell suspension (plant callus culture or animal tissue culture) may be incubated in the Evan's blue (0.1%) solution for about 15 min.
3. Take one or two drops of incubated cell suspension, cover it with cover slip and observe under microscope.
4. Score a large number of microscopic field to find out the total cells observed in the field and the number of non-stained cells (in the same field) and record.
5. Tabulate your observation and find out the total number of non-stained cells. Calculate the per cent of viable cells using the formula given above.

Observations

Use following table for recording your observation.

Sl. no.	No. of cells in a microscopic field	No. of non-stained cells
1.		
2.		
3.		
.		
.		
Total		

Results

Percent of viable cells in the given sample =

- Increase or decrease incubation period. It may vary with cell sample, e.g. plant or animal cell.

Precautions

- Incubation period is critical for accurate result.

Viva Questions

- Why is assaying cell viability important before cell culture of plant and animal cells?
- Suggest some dyes which are used for assaying cell viability.
- How can cell viability count also prove helpful in assaying pollen viability, microbial and other studies?

**Experiment 10**

Aim Plaque count by infecting the bacterium *Escherichia coli* B with its specific bacteriophage Coliphage T4.

Key Terminology

- **Capsid** a protein shell surrounding DNA or RNA in a virus.
- **Capsomers** Core composed of protein subunits surrounding DNA or RNA in virus.
- **Naked virus** (or **nucleocapsid**) Viruses consist of only capsid, i.e. DNA or RNA and protein shell.
- **Bacteriophage** Viruses that infect or attack bacteria.
- **Adsorption** Hold (molecules of gas, liquid or dissolved substances) in a layer on its surface.
- **Lytic life-cycle** The disintegration of bacteria, by rupturing the cell wall or membrane, as a result of bacteriophage infection.
- **Plaque-count** A small clear area on an agar plate, where the host bacteria have been lysed by bacteriophage.
- **Phage typing** Known bacteriophages used to identify unknown bacteria.
- **Lawn** An area (or lawn) formed by bacteria on the plate during its confluent (i.e process of merging) growth.

Basic Principle

Viruses are infectious agents with both living and non-living characteristics. They reproduce at a rapid rate, but only in living host cells. Viruses are usually much smaller than bacteria. Most are submicroscopic, ranging in size from 10–250 nm. Structurally, viruses are much simpler than bacteria. Every virus contains a genome of single-stranded or double-stranded DNA or RNA that functions as its genetic material. This is surrounded by a protein shell called a **capsid** or **core** composed of protein subunits called **capsomers**. Many viruses consist of no more than nucleic acid and a capsid, in which case they are referred to as **nucleocapsid** or **naked viruses**. Most animal viruses have an envelope surrounding the nucleocapsid and are called **enveloped viruses**. Envelope usually comes from the host cell's membranes by a process called budding, although the virus does incorporate glycoprotein of its own into the envelope.

Bacteriophages are viruses that infect only bacteria. In addition to the nucleoprotein or head, some have a rather complex tail structure used in adsorption to the cell wall of the host bacterium. Since viruses lack organelles and are totally dependent on the host cells metabolic machinery

for replication, they cannot be grown in synthetic media. In the laboratory, animal viruses are grown in animals, in embryonated eggs, or in cell (in cell culture, the host animal cells are grown in synthetic medium and then infected with viruses). Plant viruses are grown in plants or in plant cell culture. Bacteriophages are grown in susceptible bacteria.

In this experiment we will work with bacteriophages since they are the easiest viruses to study in the lab. Bacteriophages, such as Coliophage T4 will be used. It replicates by the **lytic life cycle** and are called **lytic bacteriophages**.

The life cycle of Coliophage T4 consists of the following steps:

1. **Adsorption:** Attachment sites on the bacteriophage tail adsorb to receptor sites on the cell wall of a susceptible host bacterium.
2. **Penetration:** A bacteriophage enzyme 'drills' a hole in the bacterial cell wall and the bacteriophage injects its genome into the bacterium. This begins the eclipse period (the period in which no intact bacteriophages are seen within the bacterium).
3. **Replication:** Enzymes coded by the bacteriophage genome shut down the bacterium's macromolecular (protein, RNA, DNA) synthesis. The bacteriophage genome replicates and the bacterium's metabolic machinery is used to synthesize bacteriophage enzymes and bacteriophage structural components.
4. **Maturation:** The bacteriophage parts assemble around the genome.
5. **Release:** A bacteriophage coded lysozyme breaks down the bacterial wall made of peptidoglycan causing osmotic lysis of the bacterium and release of the intact bacteriophages.
6. **Reinfection:** From 50–200 bacteriophages may be produced per infected bacterium and they now reinfect surrounding bacteria.

In this experiment we will perform two aspects. In the first part study (i) **plaque count**, and (ii) in the second part **viral specificity**.

(i) Plaque Count A plaque is a **small, clear area on an agar plate** where the host bacteria have been lysed as a result of the lytic life cycle of the

infecting bacteriophages. As the bacteria replicate on the plate they form a 'lawn' of confluent growth. Meanwhile, each bacteriophage that adsorbs to a bacterium will reproduce and cause lysis of that bacterium. The released bacteriophages then infect neighbouring bacteria, causing their lysis. Eventually a visible self-limiting area of lysis, a **plaque**, is observed on the plate.

(ii) Viral Specificity It means that a specific strain of bacteriophage will only adsorb to a specific strain of susceptible host bacterium. In fact, viral specificity is just as an enzyme-substrate reaction or an antigen-antibody reaction. Therefore, viral specificity can be used sometimes as a tool for identifying unknown bacteria. Known bacteriophages are used to identify unknown bacteria by observing whether or not the bacteria are lysed. This is called **Phage typing**.

Materials

For Plaque count

- A tube containing 9.9 ml of sterile saline
- 3 tubes containing 9.0 ml of sterile saline
- 3 sterile empty dilution tubes
- Tryptic soy agar—3 plates (*See Annexure III, No. 1*)
- Sterile 1.0 ml pipettes – 3 Nos., or Pipetman (with disposable tips)
- Sterile 10.0 ml pipette-one
- Dilution tubes
- Bottle of Melted Motility Test medium from a water bath at 47°C
- Culture of *E. coli* B (strain) in Tryptic Soy broth
- Suspension of Coliophage T4
- Incubator set at 37°C

For viral specificity

- Tryptic soy agar plates—2 Nos. (*See Annexure III, No. 1*)
- Tryptic soy broth culture of four unknown bacteria labelled #1, #2, #3 and #4
- Suspension of Coliophage T4 (For isolation of Coliophage T4 from Sewerage water see *Chapter VI, Experiment 4*)

Methods**For Plaque Count**

1. Take one tube containing 9.9 ml of sterile saline, 3 tubes containing 9.0 ml of sterile saline, and 3 sterile empty dilution tubes and label the tubes.
2. Dilute the Coliphage T4 stock as described below:
 - (a) Remove a sterile 1.0 ml pipette from the bag. Do not touch the portion of the pipette that will go into the tube and do not lay the pipette down. From the tip of the pipette to the '0' line is 1 ml; each numbered division (0.1, 0.2 etc.) represents 0.1 ml; each division between two numbers represents 0.01 ml (preferably use Pipetman).
 - (b) Insert the cotton-tipped end of the pipette into a blue 2 ml pipette filter.
 - (c) Uncap the sample of Coliphage T4, insert the pipette to the bottom of the tube, and withdraw 0.1 ml of the sample by turning the filter knob towards you. Recap the tube.
 - (d) Flame the 10^{-2} dilution tube and dispense the 0.1 ml of Coliphage T4 into the tube by turning the filter knob away from you. Draw the liquid up and down in the pipette several times to rinse the pipette and help mix. Flame and cap the tube. This will give a $1/100$ or 10^{-2} dilution of the bacteriophage.
 - (e) Using a vortex mixer, mix the tube thoroughly. This is to assure an even distribution of the bacteriophage throughout the liquid.
 - (f) Using the same pipette and procedure, aseptically withdraw 1.0 ml from the 10^{-2} dilution tube and dispense into the 10^{-3} dilution tube. This will give a $1/1000$ or 10^{-3} dilution of the bacteriophage.
 - (g) Using a vortex mixer, mix the tube thoroughly. Repeat the process (as described above) for 10^{-4} dilution from 10^{-3} and 10^{-5} dilution from 10^{-4} dilution. Ultimately this will give you a $1/100,000$ or 10^{-5} dilution.
- Discard the pipette or plastic tips after every dilution (to bio-waste container) in case you are using Pipetman.
3. Take three empty, sterile tubes and test as described below:
 - (a) Using a new sterile 1.0 ml pipette and the procedure described above, aseptically remove 0.1 ml of the 10^{-5} bacteriophage dilution and dispense into the 10^{-6} empty tube.
 - (b) Using the same pipette and procedure, aseptically remove 0.1 ml of the 10^{-4} bacteriophage dilution and dispense into the 10^{-5} empty tube.
 - (c) Using the same pipette and procedure, aseptically remove 0.1 ml of the 10^{-3} bacteriophage in each of the 3 tubes from Step 3. Using a vortex mixer, mix the tube thoroughly. Discard the pipette or plastic tips in the bio-waste container.
4. Using a new sterile 1.0 ml pipette, add 0.5 ml of *E. coli* B (strain) to the 0.1 ml of bacteriophage in each of the 3 tubes from Step 3. Using a vortex mixer, mix the tube thoroughly. Discard the pipette or plastic tips in the bio-waste container.
5. Using a sterile 10.0 ml pipette, add 2.5 ml of sterile, melted motility test medium to the *E. coli* bacterium phage mixture in each of the three tubes from Step 4. Using a vortex mixer, mix the tube thoroughly. Discard the pipette or plastic tips in the bio-waste container.
6. Quickly pour the motility medium *E. coli* bacteriophage mixture onto separate plates of tryptic soy agar and swirl to distribution of the contents over the entire agar surface.
7. Incubate the three tryptic soy agar plates right-side-up at 37°C until the next lab period.

For Viral Specificity

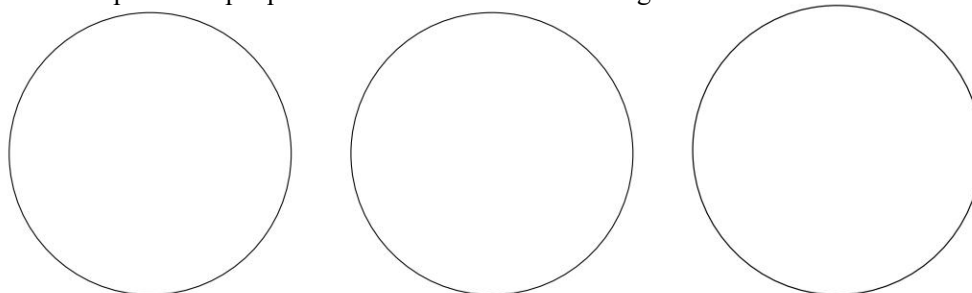
1. Using a wax marker, draw a line on the bottom of both the tryptic soy agar plates dividing them in half. Number the 4 sectors

I 12 Analytical Techniques in Biotechnology

- 1, 2, 3, and 4, to correspond to the four unknown bacteria.
2. Draw a circle about the size of a dime in the centre of the 4 sectors.
3. Using a sterile inoculating loop, streak unknown bacterium #1 on sector 1 of the first tryptic soy agar plate by streaking the loop **through the circle** you drew. **Be careful not to streak into the other half of the plate.**
4. Streak unknown bacterium #2 on sector 2 of the first tryptic soy agar plate. Likewise streak unknown bacterium #3 on sector 3 of the second tryptic soy agar plate and bacterium #4 on sector 4 of the tryptic soy agar plate.
5. Using a sterile Pasteur pipette and rubber bulb, add 1 drop of Coliphage T4 to each sector in the area outlined by the circle.
6. Incubate the 2 tryptic soy agar plates **right side up** at 37°C until the next lab period. (Also consult Chapter VII, Experiment 7).

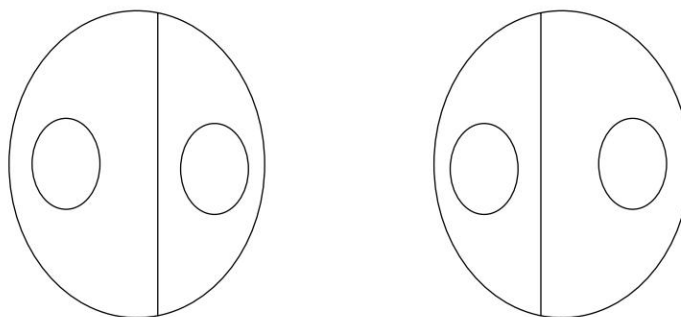
Observations**For Plaque Count**

Observe the 2 plates for plaque formation and make a drawing.



1/10,000 (10^{-4}) dilution 1/100,000 (10^{-5}) dilution 1,000,000 (10^{-6}) dilution

For Viral Specificity Make a drawing of your results and state which of the unknown (i.e. #1, #2, #3 or #4) is *E. coli*.

**Results**

- (i) Recognize plaques and state their causes.
- (ii) Interpret the result of a viral specificity test using Coliphage T4.

Precautions

- For dilution use fresh 1.0 ml pipette or plastic tips while using a Pipetman.
- Do not use your mouth for pipetting. Preferably use a Pipetman.

Viva Questions



- What is a plaque and how is it developed?
- What is phage typing?
- What is meant by lytic life cycle of a bacteriophage?
- How is specificity of bacteriophage proved in this experiment?

Notes and Sketches





4

Plant Tissue Culture



Experiment I

Aim Preparation of Stock solution for MS basal medium and Plant Growth Regulator Stock.

Key Terminology

- **Totipotency** The capacity of a cell to develop into a complete organism by regeneration.
- **Plasticity** To alter development according to the requirement, viz. environment.
- **Regeneration** Regrow new tissue or bring new and more vigorous growth of tissue.
- **Growth medium** Nutrients through which a force or other influences are transmitted for plant development.
- **Basal medium** Minimum nutrients through which base requirements of essential elements are delivered.
- **Essential elements** Chemical substances needed for plant development as a priority.
- **Organic supplement** Substance which substitutes living matter.
- **Macronutrients** See essential elements.
- **Morphogenesis** Change in the form and structure of living organisms.
- **Sterile distilled water** Water obtained from above process is taken in a flask and autoclaved at 121° for 15 min.
- **Stock solution** Ten times or higher concentration of macro-, micro- and organic nutrient than the actual requirement prepared beforehand and stored. At the time of media preparation required amount is taken with the help of pipette to give exact concentration.
- **Hardening** The process of adjusting the cultured plantlets from artificial to natural climate.

- **Heat-labile** Chemicals which are heat sensitive.
- **Membrane filter** Filters with varying pore size made up of cellulose acetate or cellulose nitrate. Membranes retain particles and microorganisms that exceed their pore size.

Basic Principle

When provided with suitable conditions each living cell of a multi-cellular organism is capable of independent development (White, 1963). Plants due to their sessile nature and long life span have developed a greater ability to endure extreme conditions and predation than animals. Many of the processes involved in plant growth and development adapt to environmental conditions. This plasticity allows plants to alter their metabolism, growth and development to best suit their environment. When plant cells and tissues are cultured *in vitro* they generally exhibit a very high degree of plasticity, which allows one type of tissue or organ to be initiated from another type. In this way, whole plant can be subsequently regenerated. This regeneration of whole organisms depends upon the concept that all plant cells can, given the correct stimuli, express the total genetic potential of the parent plant. This maintenance of genetic potential is called **totipotency**. The term “totipotency” was coined by Morgan in 1901, to describe this capacity of cell to develop into a complete organism by regeneration. Plant cell culture and regeneration do, in fact, provide the most compelling evidence for totipotency.

When cultured *in vitro*, all the needs, both chemical and physical, of the plant cell have to be met by the culture vessel, the growth medium and the external environment (light, temperature, etc.). The growth medium has to supply all the essential mineral ions (See Table 4.1.1) required for growth and development. In many cases (as the biosynthetic capability of cells cultured *in vitro* may not replicate that of the parent plant), it must also supply additional organic supplements such as amino acids and vitamins. Many plant cell cultures, as they are not photosynthetic, also require the addition of a fixed carbon source in the form of sugar (most often sucrose). One other vital component that must also be supplied is water, the principal biological solvent. Physical factors, such as temperature, pH, the gaseous environment, light

(quality and duration) and osmotic pressure, also have to be maintained within acceptable limits.

Murashige and Skoog (1962) (MS) proposed basic plant tissue culture medium which is most commonly used for plant regeneration. It provides all the compounds needed for plant growth and development, including compounds synthesized by an intact plant, but not by an isolated piece of plant tissue. Culture media used for *in vitro* cultivation of plant cells are composed of three basic components:

- (a) essential elements, or mineral ions, supplied as a complex mixture of salts
- (b) an organic supplement supplying vitamins and/or amino acids
- (c) a source of fixed carbon; usually supplied as the sugar sucrose.

Table 4.1.1 Some of the elements important for plant nutrition and their physiological function. These elements have to be supplied by the culture medium in order to support the growth of healthy cultures *in vitro*

	ELEMENT	FUNCTION
1.	Nitrogen	Component of proteins, nucleic acids and some coenzymes element required in greatest amount
2.	Potassium	Regulates osmotic potential, principal inorganic cation
3.	Calcium	Cell wall synthesis, membrane function, cell signalling
4.	Magnesium	Enzyme cofactor, component of chlorophyll
5.	Phosphorus	Component of nucleic acids, energy transfer, component of intermediates in respiration and photosynthesis
6.	Sulphur	Component of some amino acids (methionine, cysteine) and some cofactors
7.	Chlorine	Required for photosynthesis
8.	Iron	Electron transfer as a component of cytochromes
9.	Manganese	Enzyme cofactor
10.	Cobalt	Component of some vitamins
11.	Copper	Enzyme cofactor, electron-transfer reaction
12.	Zinc	Enzyme cofactor, chlorophyll biosynthesis
13.	Molybdenum	Enzyme cofactor, component of nitrate reductase

For practical purposes, the essential elements are further divided into three categories:

- (a) macroelements (or macronutrients)
- (b) microelements (or micronutrients)
- (c) an iron source.

The macronutrients are needed in millimolar (mM), whereas, micro-elements are required in much lower concentration (i.e. micro molar, μM).

As the plants in *in vitro* culture are unable to undergo photosynthesis effectively, the culture medium is supplemented with sugar. These are essential constituents of enzymes, required for metabolic activities. Vitamins are required in trace amount as they catalyse the enzyme system of the cells. The organic compounds serve as a source of carbon and energy. They are used in concentration, e.g.

20–30 g/l. Sucrose and D-glucose (carbohydrates) are commonly used; but glycerol and myoinositol are also the principal sources of carbon. Other complex organic compounds such as peptone, yeast extract, malt extracts, coconut water, tomato juice etc. are also added occasionally.

Several growth hormones are known which stimulate the biological activity in culture materials. For development of shoots and roots, plant growth regulators are supplemented at a very low concentration. In *in vitro* culture, auxin and cytokinins (e.g. Kinetine) are the two important plant growth regulators. It is essential to regulate the amount of auxin and cytokinin, for effective morphogenesis of plant tissue culture. Supplementation of these two plant growth regulators also depend on the availability of endogenous auxin in the plant tissue (See Experiment 5).

Generally, MS medium consist of 95% water, which is controlled by addition of agar-agar depending on requirement of semi-solid or liquid medium. It is always recommended to prepare fresh medium for better results. However, a concentrated stock solution is prepared in advance and added to medium as required. This saves time and labour.

Materials

- Amber bottles (for storing Stock solutions) 150 ml, 200 ml and 50 ml
- Volumetric flasks—50 ml, 100 ml, 250 ml, 500 ml, and 1 l
- Glass beakers 250 ml and 500 ml
- Pipettes—1 ml, 10 ml and 50 ml
- Glass marker
- Weighing balance
- pH meter
- Autoclave
- Chemicals as per Annexure IV
- 0.1N.HCl and KOH or NaOH

Methods

For Stock Solutions

1. For preparation of stock solutions, weigh the compounds of **Stock A** (See Annexure IV) and put it in a 100 ml volumetric flask.

Add about 50 ml of Double distilled water to slowly dissolve the compounds. When the compounds are completely dissolved, make up the volume up to the mark and shake thoroughly. Store the content in a 150 or 200 ml amber bottle and place it in a fridge after proper labelling.

2. For preparation of **Stock B** (See Annexure IV) take the required compounds in a 100 ml volumetric flask, add 50 ml of double distilled water. After dissolving make up the volume up to the mark. Mix it thoroughly in 150 ml amber bottle and transfer it in a fridge after marking it.
3. Repeat the Step 1 for **Stock D** solution.
4. For preparation of **Stock C**, take the required amount (See Annexure IV) of Na_2EDTA in a 100 ml beaker, and add about 30 ml of double distilled water. In case the sodium salt does not dissolve warm it (do not allow it to boil) for 1 h or more if necessary. Cool the dissolved solution and transfer it to 100 ml volumetric flask. Rinse the beaker for 3–4 times with small quantity of double distilled water and add it to the volumetric flask. Also dissolve Ferrous sulphate in a beaker and gradually add it to the volumetric flask, by gently stirring the flask. Make up the volume up to the mark and shake thoroughly. Store it in a fridge after properly marking it.
5. For **Stock E, F and G**, take the required content (See Annexure IV) in separate 20, 50 and 10 ml amber bottles and with the help of 10 ml, and 50 ml pipettes add 20 ml, 50 ml and 10 ml of double distilled water, respectively. Shake gently to dissolve the content. Mark it with glass marker and fridge it.
6. **Sterilization of some heat-labile chemicals**
There are many chemicals which are heat sensitive (i.e. **heat-labile**). At high temperature, e.g. **naphthalene acetic acid (NAA)**, **Gibberelic acid (GA_3)**, **enzymes**, **antibiotics** are unstable. After autoclaving the medium, it is cooled below 60°C and these chemicals are filtered through **membrane filters** with varying pore sizes (viz. 0.22, 0.45, 0.55 μM) are added to the

media. However, 0.22 μ M membrane filters are more popular. These membrane filters are commercially available (e.g. Millipore and Whatman Membrane filters). These filters are made up of **cellulose acetate** or **cellulose nitrate** and are available in pre-sterilized, plastic disposal units or as individual autoclavable units. A small quantity of media is generally passed through a membrane filter fitted with vacuum pump. If the required quantity of plant growth regulator solution, enzyme or antibiotic is very less, it may be passed through a dispensable syringe filter (0.22 μ M). The whole process is carried out in sterile condition in front of a Laminar Air-flow chamber. The stock solution may be **stored in a deep freezer** (Also consult Chapter 1; *k-Sterilization Techniques*, page 24).

For Preparation of MS Basal Media from Stock Solution

1. According to the requirement take 250 ml or 500 ml or 1 l volumetric flask. To it add the required $\frac{1}{4}$ or $\frac{1}{2}$ or same amount of Stock solution as mentioned (given for each stock solution from A to G in Annexure IV) in the flask.
2. Add cytokinin or auxin as per requirement.
3. Add sucrose at the rate of 3% (i.e. 7.5 g, 15 g or 30 g for 250 ml, 500 ml or one litre of MS basal media, respectively).
4. After sucrose is dissolved add required plant growth regulator. Make up the volume of the volumetric flask with double-distilled water. After putting a glass-stopper shake the flask properly.
5. Take it in a beaker and adjust the pH to 5.8 ± 0.2 , using a pH meter. Use 0.1 N.HCl or 0.1N.NaOH for adjusting the pH. In case the solution is below 5.8, use 0.1 N.NaOH or above 5.8 use 0.1 N.HCl. Try to adjust the pH with minimum possible HCl and NaOH.
6. Now heat it and add to it the required amount of agar-agar (shake the content with a glass rod) at the rate of 0.8% (i.e. add 2.0 g, 4.0 g or 8.0 g, respectively to 250 ml, 500 ml or 1 l of MS culture media).
7. Boil it till it starts frothing or the turbid solution becomes completely transparent throughout the beaker.
8. Cool it below 60°C and then **add heat-labile plant growth regulator** (if any). Mix the contents thoroughly. Dispense the culture media either in culture tube (20 ml each) or at the rate of 50 ml in wide-mouthed conical flask. Plug the mouth immediately with a cap or cotton plug and autoclave them at 121°C for 15 min at 15 pounds pressure per square inch.
9. Transfer the tube or flasks to the tissue culture room (Fig. 4.1.1) in a dark place and allow it to solidify. For preparing slants, lay the culture tubes at 45° angle. Culture or flasks will be ready for inoculation after few hours time.



Fig. 4.1.1 View of a typical Plant Tissue Culture Laboratory

Observations

Use the culture tubes or conical flasks for inoculation in the next lab period for experiment 2 or 3. Observe the response of the inoculated materials regularly.

Precautions

- Over-autoclaving should be avoided, it will degrade some medium components, viz. sugar and agar-agar.
- Over-boiling and incorrect pH of the medium (to be maintained at 5.8 ± 0.2) may disturb the solidification of agar-agar.

- Ensure that water level of the autoclave and air-exhaust is functioning properly.
- Autoclave screws should be properly closed.
- All stock solution and dilutions should be prepared in double distilled water and marked properly with date of preparation.
- As soon as, Stock A, D or F shows white thread like growth (fungal mycelia growth), it should be rejected. Preferably stock solutions should be used up within a month.

Viva Questions

- What is the advantage of preparing stock solutions?
- How should heat-labile plant growth regulators be added to the culture medium?
- What other culture media are generally used for plant tissue culture?
- Suggest some alternative chemicals as nitrogen source in the MS basal media.
- Why is pH critical for the medium?

**Experiment 2****Aim**

Aseptic culture technique for establishment and maintenance of carrot root cubes and Tobacco plant by leaf disc culture.

Key Terminology

- **Cell culture** The growing of cell *in vitro*, including the culture of single cell or small aggregates of cell in a liquid medium.
- **Explant** The tissue taken from a plant or seed and transferred to a culture medium to establish a tissue culture system or regenerate a plant.
- **Meristematic** See Chapter II Experiment 1.
- **Aseptically** Without being contaminated by bacteria, viruses, fungi, mycoplasma and other competing microorganisms in cultures of eukaryotic cells or tissue.
- **Differentiation** Separation of initial cell into different distinguished cell lines during cell growth.
- **Parenchyma** Functional tissue of an organ as distinguished from the connective and supporting tissue.
- **Proliferating** Increasing rapidly in number.
- **Sub-culture** Transfer of previous cultures to fresh medium and maintain the continuity of the culture.

- **Hardening** See Experiment 1.
- **Acclimatization** Make accustomed of cultured plantlets to a new climate or new condition.
- **Desiccation** Removal of moisture from the plantlets transferred from a culture condition to the natural atmosphere.
- **In vitro** Any process carried out in sterile cultures, or the measurement of biological processes outside the in-vivo organism such as enzyme.
- **Membrane filter** See Experiment 1.

Basic Principle

In vitro cloning of plant cells and tissues is encouraged as they are totipotent. The capacity to propagate genetically identical plants from a cell or tissue of the parent plant has proved and fulfilled many possibilities.

Millions of new plants may be **micro-propagated asexually** by culturing a single part aseptically. **Explants** (a piece of plant tissue) of **meristematic** (dividing tissues) **cells** more rapidly give rise to basic morphological units,

i.e. roots, stems and leaves. Parenchyma is the most versatile of all types of tissues. They are capable of division and growth. Development of a tissue is characterized by three types of cellular activities, viz. cell division, cell elongation and cell differentiation. Therefore, explants from healthy and young parts (meristematic shoot tip) of the plant are used. Parenchyma cells have an excellent morphogenetic potential. Parenchyma from stems, rhizomes, tubers, and roots are easily accessible and will generally respond quickly to culture conditions *in vitro*. According to Murashige (1974), the whole procedure of development by micro-propagation may be divided into four different stages:

Stage I *Establishment of explants aseptically.* In this stage a piece of plant tissue is trimmed into a proper size (not less than 2 mm² or mm³), disinfected (i.e. removal of surface contamination) and aseptically (without getting contaminated by bacteria or fungi) transferred to the culture media.

Stage II *Multiplication of propagules by repeated sub-cultures on a specific nutrient medium.* Depending upon the requirement, explants is induced to produce vegetative shoots (multi-shoot) or callus by supplementing the nutritional composition of medium by auxins (viz. BAP, NAA, 2,4-D etc.). This stage requires repeated subculture for multiplication and growth of culture. A **callus is an amorphous mass of loosely arranged thin walled parenchyma cells developing from proliferating cells of the parent tissue** (Fig. 4.6.1). However, when callus is induced, it requires supplementation of subculture with auxin for inducing shoots. Sub-culturing for shoots should not be encouraged more than 3 to 4 times. Along with number of sub-culture there is reduction in the number of shoots.

Stage III *Rooting.* At this stage rootless shoots (i.e. apical dominance) are cultured in the medium to induce adventitious roots by auxin supplementation.

Stage IV *Hardening of plantlets and planting into soil.* In this stage plantlets growing in an

artificial environment are brought out of culture tubes for acclimatizing them in natural harsh environment. Control of humidity is the most important factor. Different soil mixtures are used to suit the plantlets. At this stage plantlets dehydration (from 100% humidity of tubes to low humidity) and invasion of microorganisms and become independent (by photosynthesis).

In this experiment we will make use of the MS basal media prepared in the last experiment. However, cytokinin and auxin necessary for carrot roots and tobacco leaf discs for regeneration will be added along with the MS media.

Materials

- Plant materials—Fresh carrot roots and healthy fresh meristematic tobacco leaves
- Petri-dishes
- Beakers 500 ml
- Sterilized blades (with holder) or cork borer
- Sterile forceps
- 0.1% mercuric chloride solution
- Tween-20
- 2,4-D (3mg/l)
- NAA and BAP (1mM)
- Membrane filter
- Double distilled water
- Sterile filter paper

Methods

For Carrot Cubes

1. With the sterile blade cut the carrot roots into 0.2 or 0.4 mm cubes which should include the parenchyma layer.
2. Take the carrot cubes in a petri-plate containing 1.0% Tween-20 solution and wash the cubes for 2–3 min by frequent shaking. Transfer them into running water for 5 min.
3. Wash the cubes with distilled water and put them in 0.1% mercuric chloride. Leave them for 2–3 min and then transfer to distilled water. After washing for 4–5 times in distilled water, leave them in distilled water for 10–15 min for desiccation of mercuric chloride.

4. For inducing callus in carrot root cubes, supplement MS basal media (*See Experiment 1; Methods, Step 2*) with 3 mg/l of 2,4-D (during preparation of MS media), and prepare 45° slants.
 5. With the help of sterile forceps transfer the cubes from distilled water to a dry petri-plate (if necessary soak them with sterile filter paper).
 6. With the help of sterile forceps transfer one carrot root cube in a culture tube (*See Fig. 3.2.1 in Chapter 3*).
 7. Transfer the inoculated culture tubes in a tissue culture room (place the tubes at 45° slanting position), maintained at $24 \pm 2^\circ\text{C}$ with white fluorescent light in 16 h light and 8 h dark regime.
 8. Change the culture tubes with freshly prepared media after every 3 weeks interval.
- min. After that transfer the discs to a petri-plate or place them over a sterile filter paper.
4. For shoot multiplication, supplement MS basal media with 2.5 μM of BAP and 0.5 μM of NAA (Add filter sterilized NAA after autoclaving MS media and cooling it below 60°C).
 5. With the help of sterile forceps (put a small sterile cotton swab around the forceps tips) transfer one disc in one culture tube. Follow steps 6 to 8 as mentioned for carrot cubes.
- NOTE:** For experience, practice transfer of explants in blank slant tubes or conical flasks. Add 0.6% agar to distilled water and boil. Dispense 15 or 20 ml of the content in culture tubes or flasks. Allow it to solidify before practice.

Observations

- (a) Observe the culture periodically. Discard the cultures showing infection (Fungal growth or blackening of explants due to mercury poisoning or media becoming brown due to release of phenolic compounds by the tissue).
- (b) Observe changes for 4 to 6 weeks from 3rd week onward at an interval of one week.
- (c) Also record how many culture tubes were inoculated and how many of them discarded, so that you can calculate percent of successful transfer of cultures in carrot root cubes and tobacco leaf discs.

For Tobacco Leaf Discs

1. Using sterile cork-borer (4 mm diameter) punch out several leaf discs from a meristematic leaf (or use sterilized blades to trim the leaf into 4 mm square size).
2. Wash the leaf discs in distilled water 3–4 times and dip them in 0.1% mercuric chloride for 1–2 min for surface sterilization.
3. Wash the discs several times with distilled water and leave them in distilled water for 10

Results

Period of observation	Changes in Carrot root cubes	Changes in Tobacco leaf discs
3 rd Week		
4 th Week		
5 th Week		
6 th Week		
7 th Week		
8 th Week		

Precautions

1. Increase or decrease disinfecting period with mercuric chloride depending on infection and blackening of tissue.
2. During transfer of explants with forceps, take care that the carrot cubes or tobacco discs are not damaged.
3. Add membrane filtered NAA after autoclaving of MS basal media and cooling it below 60°C.
4. In case media becomes brown or yellowish in carrot culture tubes, frequently sub-culture or lower light intensity to reduce exudation of Phenolic compounds.

Viva Questions

1. Why media turns brown in colour?
2. What is the role of 2,4-D and NAA on carrot root cubes and tobacco leaf discs, respectively?
3. How you can prove that explants have been aseptically transferred?
4. Why is it necessary to membrane filter NAA?
5. Why is it necessary to include parenchyma layer in carrot cubes?

**Experiment 3****Aim**

Aseptic organ culture technique, of embryo and ovary.

Key Terminology

- **Organ culture** The maintenance or growth of organ primordial or whole parts of an organ *in vitro*, in a way that allows differentiation and/or preservation of its structure and/or function.
- **Somatic embryoids** A plant regeneration method *via* direct somatic embryogenesis achieved from cotyledon explants, like their zygotic counterparts.
- **Embryo-rescue** Application of embryo culture techniques to obtain inter-specific hybrid plants prior to abortion of the embryo due to post-fertilization hybridization barriers.
- **Incompatibility** See Chapter II; Experiment 5.
- **Multi-shoots** During callus regeneration, it initiates number of shoot-lets, which ultimately give rise to independent shoots or plantlets.
- **Shoot primordial** Beginning or initiation of shoot initials from a callus culture.
- **Inherent deficiency** A fundamental flaw in the discretionary access control mechanism.

- **Callus induction** Use of plant growth regulators to force an unorganized growth of plant cells *in vitro* on a culture medium.

Basic Principles

Embryos can be used as explants to generate callus cultures or somatic embryos. Both immature and mature embryos can be used as explants. Immature embryo derived embryogenic callus is the most popular method of monocot plant regeneration.

The culture media on which organ is cultured are the same as described for cell and tissue culture. However, organ culture technique demands more tactful manipulation than general tissue culture. In addition to root, shoot and pollen culture, embryo and ovary culture has been practised for the production of haploid plants or recovery of plants from distinct crosses. Embryo or ovary culture is useful where embryo or ovary fails to develop due to degeneration of embryonic tissues. Embryo culture is a routine technique employed in orchid propagation and in breeding of those species that show dormancy. In some species, viz. tea, shoots are regenerated via the embryonic callus. The embryo callus produces **somatic embryoids**.

However, it takes 8 minimum weeks to develop embryoids which are sub-cultured in a different medium to differentiate into buds after 2 weeks. It takes 12–16 weeks to develop multi-shoots with 4–6 leaves.

Viable hybrids are produced as a result of sexual distant crosses between two varieties of the same species of the genus or between two different genera. Production of fertile viable seeds of hybrid is rather difficult because of several barriers arising either during pollination, fertilization or embryogenesis. It has been observed that in some cases in spite of successful pollination and fertilization, the embryo does not develop. This is due to inherent deficiency or incompatibility between the developing embryo or endosperms. In such cases, immature embryos or complete ovary are dissected out from the fruit (seed) and grown *in vitro* on medium which differentiate into shoot, root and plantlets. This technique of growing immature embryo or ovary is termed as **embryo rescue**.

In this experiment we will attempt to culture groundnut embryo. This experiment will clearly show the four stages of plant micro-propagation as mentioned in Experiment 2.

Materials

- MS stock solutions (See Annexure IV)
- Casein hydrolysate (0.0; 0.2, 0.4, 0.6, 0.8 and 1.0 mg/l)
- 2,4-D (1, 2, and 4 mg/l)
- BAP (2, 4 and 6 mg/l)
- Petri-plates and cultured tubes
- Sterile forceps and sterile surgical blade
- Fresh groundnuts
- Tween-20
- Mercuric chloride (0.01%)

Method

The general method of embryo culture is as follows:

1. Take fresh and healthy groundnuts and wash them thoroughly in running water for about an hour.
2. Sterilize the surface with 0.1% Tween-20 for 15 min, rinse the shell several times with

distilled water. Break open the groundnut shell and treat the seeds with 0.01% Mercuric chloride solution for 10–15 min. Finally rinse it six times with sterile distilled water.

3. Remove the soaked seed coat aseptically and remove the cotyledons so that intact embryo can be recovered (soak the seeds in distilled water for several hours, which will help in isolating the embryo without injuring it).
4. Prepare MS basal media; supplement it with different combinations and concentrations of sugar, auxin and cytokinin for callus proliferation.
 - (a) **For callus induction:** 1 to 4 mg/l of 2,4-D along with 0.2 to 1.0 mg/l of Casein hydrolysate;
 - (b) **For shoot regeneration:** 2 to 6 mg/l of BAP; and
 - (c) **For root regeneration:** Only MS basal media without any adjuvants.
5. Inoculate the embryo without any injury with a forceps (insert the pointed end slightly into the media) and incubate the cultures at $25 \pm 2^\circ\text{C}$ under a 16 h photoperiod of 2,000 lux luminous intensity.
6. After two weeks of inoculation the embryo begins to swell on callus proliferation medium. Observe the combination of 2,4-D and casein hydrolysate that shows best and distinct callus growth. Sub-culture after every 4 weeks of interval.
7. After 8 weeks of inoculation transfer the callus on a shoot regeneration medium. Within 4 weeks of transfer into second medium the callus turns green and produces a soft spongy tissue. Some of these tissues differentiate into **embryoids**.
8. The embryoids produce a cluster of **budlets** when sub-cultured onto the same medium. The budlets grow into shoots and produce 2–3 leaves within 12 weeks.
9. Individual shoots are separated with the help of surgical blades and then sub-cultured into a fresh medium with the same composition until the shoots are well developed.
10. After 16 weeks transfer the shoots to MS basal media without any adjuvants for root regeneration.

11. When the plantlet shows sufficient root development, transfer them for acclimatization.

Observations

Record your observations for:

- Which combination (i.e. 6 concentrations of Casein hydrolysate $\times$ 3 concentrations of 2,4-D = 18 combinations) showed best callus production.
- Which concentration of BAP (out of three) showed maximum number of shoots? Also find out the number of shoots per embryo.
- Which group of shoots showed best root development, and
- Which group of plantlets was best or showed maximum acclimatization.

Viva Questions



- What are the four stages of plant micro-propagation?
- What is the function of BAP and what other cytokinin can be added in place of BAP?
- What is “embryo rescue”?
- When can embryo culture be useful?



Experiment 4

Aim

Determine callus growth and calculate the Callusing Index.

Key Terminology

- **Callus index** An indicator or magnitude with which callus growth or development occurs.
- **Habituation** It is a phenomenon of normal plant cells grown in culture which often become hormone-independent spontaneously.
- **Metabolic rate** Speed with which chemical processes in a living organism produce food used for tissue growth or energy production.
- **Cell-suspension** A mixture in which nutrient particles are dispersed throughout a fluid for cell growth or culture.
- **Semi-solid medium** A medium containing lower per cent of agar-agar (e.g. less than 0.8%) which do not allow the medium to completely solidify.

Results

From your observation and record state which particular combination showed best performance and gave the highest recovery of plants.

Precautions

- Sub-culture or transfer 4 weeks' old cultures to a fresh medium.
- Discard cultures showing infection at any stage of the experiment.
- Number of Casein hydrolysate, 2,4-D and BAP concentrations are tentative. May be reduced or increased according to the facilities available.
- Carefully mark all the combinations.

- **Transformation protocol** Procedure to transfer and incorporate DNA, especially recombinant DNA, into a cell. Cells plants or progeny resulting from this process are said to be transformed upon demonstration of the expression in the recipient organism of unique marker genes carried by the transformed DNA.
- **Regeneration** Re-grow new tissue, or bring new and more vigorous life, to an area of cell initiation.

Basic Principle

When any explant is cultured in an appropriate medium, usually with both an auxin and a cytokinin, it give rise to an unorganized, growing and dividing mass of cells called **callus**. It is usually composed of unspecialized parenchyma cells developing

from proliferating cells of the parent tissue (Dodds and Roberts, 1985). During callus formation there is some degree of differentiation (i.e. the changes that occur during development and specialization are, to some extent, reversed), both in morphology and metabolism (i.e. most of the plant cultures lose the ability to photosynthesise). During induction, the metabolic rate of cells is stimulated, duration of which depends on physiological status, and nutritional and environmental factors. Due to increased metabolic rate, cell accumulates high contents of nutritional composition and other factors which help to divide to form many number of cells. Sometimes callus of different colour appears, e.g. yellow, white, green or red. Callus culture is often performed in the dark, as light can encourage differentiation of the callus. An alternate dark and light regime of 12 h may encourage callus. During long-term culture, the culture may lose the requirement for auxin and/or cytokinin. This process is known as **habituation**, which is common in callus cultures from some plant species, e.g. sugarbeet.

Callus cultures come under one of the two categories, viz. **compact** or **friable**. In a compact callus the cells are densely aggregated, whereas in a friable callus the cells are only loosely associated with each other and the callus becomes soft and breaks apart easily. These friable calluses are of importance and provide the inoculum to form **cell-suspension** cultures. Explants from some plant species or particular cell types tend not to form friable calluses, making cell-suspension initiation difficult. However, the friability of calluses can sometimes be improved by manipulating the medium components or sub-culturing. It may also be improved by culturing in a semi-solid medium (low concentration of agar). When friable callus is placed in conical flasks into a liquid medium (same composition as the solid medium used for callus culture), under correct condition and agitated, it results in dilution of the suspension and the initiation of another batch of growth cycle.

Callus cultures are extremely important in biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of

shoots, roots or somatic embryos from which whole plants can subsequently be produced. A simplified scheme for the integration of plant transformation protocols has been represented in Fig. 4.4.1. The explants can be initiated into a variety of culture types, depending on the explants used. Regeneration by either organogenesis or somatic embryogenesis results in the production of whole plants. Different culture types and regeneration methods amiable to different transformation protocols are used depending on the plant species and culture being used. In other species there is a possibility of effective choice over culture type and/or regeneration methods, which may limit the transformation protocols that are applicable.

In culture, this proliferation can be maintained more or less indefinitely, provided that the callus is sub-cultured on to fresh medium periodically with the same composition. When grown on nutrient medium nearly for three weeks, it becomes essential to sub-culture on a fresh medium for maintaining its growth. Rate of callus growth may be measured by calculating the ratio between **dry weights** divided by **fresh weight**, which is known as the **Callusing Index**, at a fixed interval of time.

In this experiment we will induce a callus with the help of three concentrations of 2,4-D and calculate the callusing index from the carrot explants.

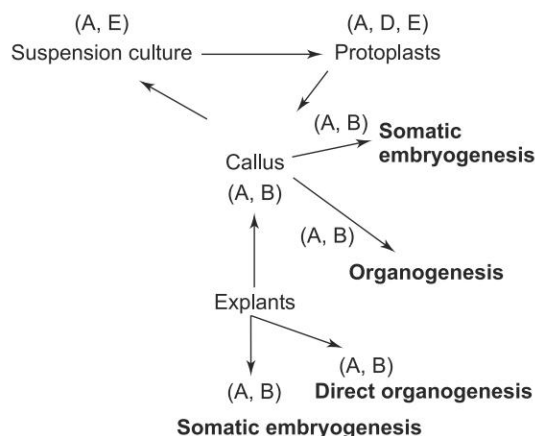


Fig. 4.4.1 The transformation protocols shown in this figure are—(A) *Agrobacterium* mediated; (B) Biolistic transformation; (D) Direct DNA uptake; and (E) Electroporation (Walden and Wingender, 1996)

Materials

- MS Stock solutions (*See Annexure IV*)
- Fresh carrot roots
- Petri-plates, culture tubes and 100 ml wide mouthed conical flasks
- Sterilized blades (with holder) and forceps
- Tween-20
- 0.1 Mercuric chloride
- Single pan weighing balance
- Hot air oven-maintained at 60°C
- 2; 3 and 4 mg/l of 2,4-D
- Sterilized distilled water
- Sterile filter paper
- Absolute alcohol
- Glass marker.

Methods

1. Follow the same steps of methods from No.1 to 6 as in this chapter (Experiment 2). Inoculate at least 9 to 12 culture tubes/flasks for each concentrations of 2,4-D.
2. Transfer the culture tubes in a tissue culture room, maintained at $24\pm 2^\circ\text{C}$ with white fluorescent 1,100 lux luminous in 12 h light and 12 h dark.
3. Observe the culture periodically and discard the cultures showing infection or discolouration of explants (due to mercury poisoning) or browning of medium (due to release of phenolic compounds by the explants).
4. Sub-culture in freshly prepared media after three weeks (21 days) interval in the same media composition. Sub-cultures should be done at least thrice (i.e. after 21; 42 and 63 days).
5. In case the volume of callus cultures is higher than the mouth of the culture tubes, transfer them to a wide-mouthed conical flask.
6. During each sub-culture take out cultures from 2 or 3 tubes and measure the fresh weight of the callus for calculating the Callus Index.
7. Take the weight of callus in a petri-dish and allow it to dry in a hot air oven maintained at 60°C. Weigh the dry callus at an interval of 2 days, till the weight is constant.

Observations

Keep a record of the initial weight of the fresh callus and final weight (after drying) of dry callus for each 2,4-D concentrations after 21, 42 and 63 days.

Results

Record your observations in the table given below:

Culture duration (in days)	Concentration of 2,4-D	Weight of callus		D/F ratio
		Fresh weight (F)	Dry weight (D)	
21 days*	2mg/l			
	3 mg/l			
	4 mg/l			

*Make similar tables for 42 and 63 days.

Also find out: (a) the trend of graph observed after drawing it. (b) which concentration of 2,4-D is best for producing callus.

Figure

Plot three separate graphs (or combine together) for three concentrations of 2,4-D, using Callus Index (D/F) values along the Y-axis and durations along the X-axis.

Concentrations of 2,4-D mentioned in this experiment are tentative therefore, according to the available facility number of concentrations may be increased or decreased. Higher number of concentrations will reveal actual trend of the graph. However, minimum three points are necessary for a graph.

Precautions

- The weight of the carrot root cubes (or explants) should be same.
- Use glass marker to label the culture tubes/conical flasks mentioning 2,4-D concentration, date of inoculation and number of sub-culture.
- For calculating the Callus Index, take an average of 2 to 3 observations (or more).

Viva Questions

- What are the changes that take place in callus culture?
- What are the uses of callus culture in plant biotechnology?
- Explain how callus develops.
- What type of tissue do we generally find in a callus?
- What is Callus Index?
- What is the importance of a friable callus?
- What is the source of cell-suspension?
- Why do callus cultures prefer dark than light?
- What are the two categories of callus culture observed in a plant tissue culture?

**Experiment 5****Aim**

Study the effect of auxin or cytokinin and other plant growth regulators.

Key Terminology

- **Plantgrowth regulators** Plant hormones which stimulates growth in animal and plant cells.
- **Auxin** A group of plant growth regulators which stimulates shoot elongation.
- **Cytokinin** Plant growth hormones which promote cell division and regulate growth and development.
- **Plant hormones** A substance produced by plants (or living things) and transported in tissue fluids to specific cells or tissues to stimulate them in action.
- **Kinetin** A kind of cytokinin, a class of plant hormone that promotes cell division. Also known as N6-furfuryladenine.
- **Endogenous** Relating to an internal cause or origin. Contrary to exogenous.

Basic Principle

We have already briefly considered the concepts of plasticity and totipotency in Experiment 1. Due to this plasticity and totipotency, specific media manipulation can be used to direct the development

of plant cells in culture. Plant Growth Regulators (PGRs) are the critical media components in determining the developmental pathway of the plant cells. The PGRs used commonly are plant hormones or their synthetic analogues. There are five classes of PGR used in plant cell culture, viz.

1. Auxins
2. Cytokinins
3. Gibberellins
4. Absciscic acid
5. Ethylene

1. Auxins These promote both cell division and cell growth. The most important naturally occurring auxin is IAA (Indole 3-acetic acid), but its use in plant cell culture media is limited because it is unstable in the presence of heat and light. It is more common, though, to use stable chemical analogues of IAA as a source of auxin in plant cell culture media. 2,4-Dichlorophenoxy acetic acid (2,4-D) is the most commonly used auxin and is extremely effective in most circumstances. Other auxins available are given below (Table 4.5.1) and some may be more effective or potent than 2,4-D in some instances.

Table 4.5.1 Commonly used auxins, their abbreviation and chemical name.

ABBREVIATION/NAME	CHEMICAL NAME
1. 2,4-D	2,4-dichlorophenoxy acetic acid
2. 2,4,5-T	2,4,5-trichlorophenoxy acetic acid
3. Dicamba	2-methoxy-3,6-dichloro benzoic acid
4. IAA	Indole-3-acetic acid
5. IBA	Indole-3-butyric acid
6. MCPA	2-methyl-4-chlorophenoxy acetic acid
7. NAA	1-naphthyl acetic acid
8. NOA	2-naphthyl oxy acetic acid
9. Picloram	4-amino-2,5,6-trichloropiocolinic acid

2. Cytokinins Cytokinins promote cell division. Naturally occurring cytokinins are a large group of structurally related (they are purine derivatives) compounds. Of the naturally occurring cytokinins, two have some use in plant tissue culture media. These are 2iP and Zeatin. Their use is not widespread as they are expensive and relatively unstable. The synthetic analogues, kinetin and BAP, are therefore used more frequently. Non-purine-based chemicals, such as substituted phenylureas, are also used as cytokinins in plant cell culture media. These substituted phenylureas can also substitute for auxin in some culture system.

Table 4.5.2 Commonly used cytokinins, their abbreviation and chemical name

ABBREVIATION/NAME	CHEMICAL NAME
1. BAP ^a	6-benzylaminopurine
2. 2iP ^b	{N ⁶ -(2-isopentyl)adenine}
3. Kinetin ^c	6-furfurylaminopurine
4. Thidiazuron ^c	1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea
5. Zeatin ^b	4-hydroxy-3-methyl-trans-2-butenylaminopurine

^a Synthetic analogues; ^b Naturally occurring cytokinins;

^c A substituted phenylurea-type cytokinin

3. Gibberellins There are numerous, naturally occurring, structurally related compounds termed

‘gibberellins’. They are involved in regulating cell elongation, and are agronomically important in determining plant height and fruit-set. Only a few of the gibberellins are used in plant tissue culture media, GA₃ being the most common.

4. Absciscic acid Absciscic acid (ABA) inhibits cell division. It is most commonly used in plant tissue culture to promote distinct developmental pathways such as embryogenesis.

5. Ethylene It is a gaseous, naturally occurring, PGR most commonly associated with controlling fruit ripening in climatic fruits, and its use in plant tissue culture is not widespread.

There is some considerable difficulty in predicting, the effects of PGR. This is because of the great differences in culture response between species, cultivars and plants of the same cultivar grown under different conditions. Moreover, endogenous concentration of genetically controlled synthesis of PGRs is responsible for differential response. However, some general principles hold true and have become general practice in most of the plant tissue culture on which its uses are based. Auxins and cytokinins are the most widely used PGRs in plant tissue culture and usually used together. The ratio of the auxin to the cytokinin determining the type of culture established or regenerated (Fig. 4.5.1). A **high auxin to cytokinin ratio generally favours root formation**, whereas a **low auxin to cytokinin ratio favours shoot formation** and an **intermediate ratio favours callus production** (Also refer Experiment 4).

In this experiment we will observe the effect of auxin and cytokinin on carrot root cubes.

Materials

- MS Stock solutions (*See Annexure IV*)
- Fresh carrot roots
- 2,4-D 0(Control); 1; 2; 3 and 4 mg/l
- BAP 0(Control); 0.2; 0.4; 0.6 and 0.8 mg/l
- 0.1% mercuric chloride
- Tween-20
- Double distilled water
- Sterilized forceps and blades (with holder)
- Culture tubes and petri-plates
- Glass marking pen

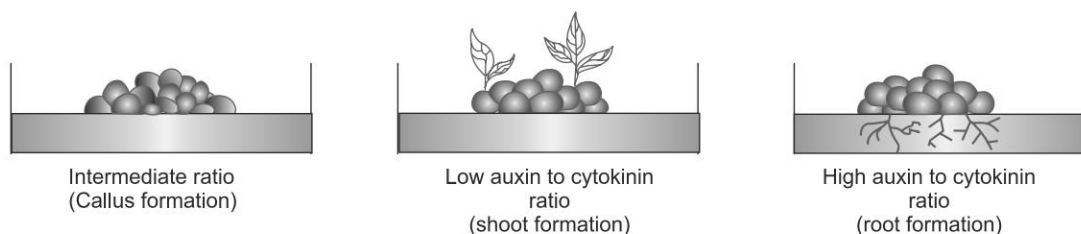


Fig. 4.5.1 Auxin to cytokinin ratio showing callus, shoot and root formation

Methods

1. With a sharp blade trim the carrot root into 0.5 or 0.6 mm cubes along with the parenchyma layer
2. Transfer the cubes in running water for 5 min.
3. Take them in a petri-plate containing 1% Tween-20 solution and wash the cubes for 2–3 min by frequently shaking the plates.
4. Wash the cubes in distilled water and further trim the cubes with sterile blade to 0.4 mm size. Put them in 0.1% Mercuric chloride for surface sterilization. Leave them for 2–3 min and then transfer to distilled water. After washing 4–5 times in distilled water, leave them in sterile distilled water for 10–15 min for removal of mercuric chloride. Remove the cubes from sterile distilled water to a dry petri plate.
 - (a) Prepare one litre of MS basal media (See *Experiment 1, Annexure IV*); Supplement with 0 (control); 2; 3; and 4 mg/l of 2,4-D and 0 (control); 0.2; 0.4; 0.6 and 0.8 mg/l of BAP. Also prepare different

auxin and cytokinin combinations viz. $(5 \times 5 = \text{Total 25 combinations} - 10 \text{ single combinations}) = 15 \text{ combinations}$ of auxin + cytokinin. Mark them with glass marking pen after dispensing them in culture tubes.

- (b) Inoculate one cube in one culture tube with sterile forceps. Incubate the tubes in tissue culture room at $24 \pm 2^\circ\text{C}$ under 16 h photoperiod of 1,600 lux luminous intensity and 8 h dark.
- (c) Observe the cultures periodically (initially alternate days) and discard the infected tubes. Sub-culture the tubes after 3 weeks of interval. Find out the differences in different combinations of auxins and cytokinins.

Observations

Record your observations for

- (a) Effect of 2,4-D and BAP alone;
- (b) Particular combination, i.e. 15 combinations of auxin and cytokinin showing (i) only callus; (ii) only shoot; (iii) callus + shoot regeneration; (iv) callus + root; (v) only root regeneration and (vi) callus + shoot + roots.

Results

Record your observation by +ve and –ve sign (i.e. + = Yes; and – = No) in the following table:

Concentrations of Auxin and Cytokinin	Only Callus	Only shoots	Callus + shoot	Callus + root	Only root	Callus + Shoots + Roots
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Precautions

- Concentrations of auxin and cytokinin mentioned in this experiment are tentative. Therefore, it may be increased or decreased according to the facilities available.
- Carefully mark all the combinations.
- Initially discard the cultures showing infection.
- Include parenchyma layer in the carrot cubes.

Viva Questions

- What should be the auxin: cytokinin ratio showing callus, shoot and root regeneration?
- What are the uses of gibberellin in plant tissue cultures?
- How many different classes of PGRs are usually used in plant tissue culture?
- Which auxin is thermal and light sensitive?
- For inducing callus which particular auxin will you prefer to use in the media.

**Experiment 6**

Aim Micro-propagation of rice by *in vitro* organogenesis from embryo

Key Terminology

- **Organogenesis** The process of initiation and development of a structure which shows natural organ form and/or function, the initiation of which is temporally separated or *de novo* in origin.
- **Primordium** Is defined as an organ or tissue in its earliest recognizable stage of development.
- **Meristemoid** The meristem is a dome of actively dividing cells (about 0.1 mm in diameter and 0.25 mm long) having main shoot and axillary buds.

Basic Principle

Organogenesis, i.e. development of organ, viz. root, shoot and leaves that are induced in plant tissue culture from embryo. Since embryo is an independent structure and does not have vascular tissue, it is not supposed to be a plant organ. Organogenesis can be regulated by a balance between cytokinin and auxin. It has been demonstrated that a **high** ratio of auxin : cytokinin stimulated the formation of root in tobacco callus, but a low ratio of the same induced shoot formation. Torrey (1966) suggested the hypothesis of organogenesis, that organogenesis in callus starts with the development of a group of meristematic cells, i.e. meristemoids can respond to the factors within the system to initiate a primordium which induces either root or shoot depending on kind of factors.

In vitro control of organogenesis by auxins and cytokinins.*

Auxin (in mg/l)	Cytokinin (in mg/l)	Organogenesis
0.0	0.2	No growth
0.03	1.0	Shoots
3.0	0.02	Roots
3.0	0.2	Callus

* Nandi and Palni (1992)

Some plants are regenerated *via* callus phase which is termed “indirect regeneration”. First, callus is induced from explants, an unorganized growth of dedifferentiated cells. Some of the callus reorganizes to form **meristemoid**, which is very similar to meristem tissue.

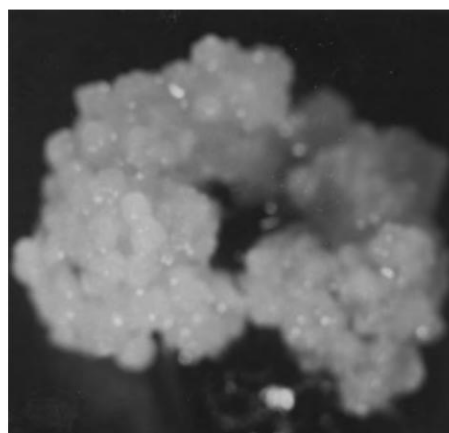


Fig. 4.6.1 White-friable, globular callus

In this experiment we will use MS basal media supplemented with 2,4-D to induce callus, out of which whitish-friable calli (Fig. 4.6.1) will

be selected for redifferentiation with MS basal media supplemented with cytokinin (BAP). Calli showing healthy growth having green-spots will be selected and sub-cultured in fresh media. After regeneration of shoots, it will be transferred to root induction media. This experiment will clearly show the four stages of plant micro-propagation as mentioned in Experiment 2.

Materials

- MS-Stock solutions, (See Annexure IV)
- Glassware and pipettes for media preparation
- 2,4-D (2.0 mg/l)
- BAP (3.0 mg/l)
- Casein hydrolysate (0.2 to 1.0 mg/l)
- Petri-plates
- Rice seeds (var. *Indica* and N6 for *Japonica*)
- Sterile distilled water
- Tween-20
- Sterile filter paper

Method

For Callus Induction

1. Prepare 500 ml of MS basal media (as per Experiment 1, Chapter 4). Supplement it with 2.0 mg/l of 2,4-D + 0.0; 0.2; 0.4; 0.6; 0.8 and 1.0 mg/l of casein hydrolysate.
2. Dispense the sterile media in sterile petri-plates (20 to 25 ml each), wrap the plates with paper and autoclave them. Transfer them to laminar hood. Allow them to solidify.

Preparation of Plant Material

3. Take the desired variety of rice seeds and carefully remove the lemma and palea with forceps without damaging the embryos.
4. Select healthy and shiny seeds. Take them in a sterile flask and surface sterilize with 70% absolute alcohol for 1 or 2 min. Rinse them 3 times with sterile distilled water.
5. Sterilize the seeds with 50% Chlorox for 25–30 min (preferably under vacuum or constant shaking). Add a drop of surfactant, e.g.

Tween-20, to enhance the effect of Chlorox.

6. Wash the seeds 3–5 times with sterile distilled water to remove all the Chlorox. Make the seeds dry with sterile filter paper.
7. Remove the paper cover from the petri-plates and place 10–15 seeds with sterile forceps after switching on the laminar flow.
8. Incubate the plates in dark for 30–40 days, check the cultures for contamination after every 3rd day and then once a week.

Selecting Calli for Organogenesis

9. The embryogenic calli that are white, globular, friable (Fig. 4.6.2), and dry without showing any differentiation (i.e. without shoot or root like structure) are selected for further regeneration. Expose the culture-plates to light (See Experiment 4; Chapter 4).



Fig. 4.6.2 *In vitro* organogenesis of rice from embryo

10. Transfer the selected healthy embryogenic calli to MS basal media supplemented with 3 mg/l of BAP.

Regeneration and Rooting

11. Subculture the proliferating calli showing green-spots into a conical flask (100 or 250 ml,

- wide-mouthed) containing fresh regeneration media with same supplementation.
12. After 4 weeks, when the shoots are 3–4 cm long, transfer them to MS basal media (without any auxins or cytokinin) for root development. Also try $\frac{3}{4}$ or $\frac{1}{2}$ diluted MS-medium for inducing roots.
 13. When sufficient root develops, put the plantlets for acclimatization.
 14. Plantlet roots are thoroughly washed (without injuring them) and transferred to pots for hardening.
- (b) Number of shoots regenerated per rice seed
 - (c) Record number of shoots, successfully developed roots
 - (d) Number of plantlets acclimatized and find out the number of plants recovered per rice seed

Precautions

- Use fresh callus for regeneration of shoots. Old or mature callus are not suitable for regeneration.
- Selection of rice variety *Indica* or N6 *Japonica* is very critical, as all varieties do not respond to organogenesis.
- Take care that the embryo of the rice seeds should touch the media.

Observations

Keep records for following

- (a) Number of rice seeds which showed callusing

Viva Questions



- What is organogenesis?
- What is meristemoids?
- What happens when the auxin : cytokinin ratio is high?
- What happens when the auxin : cytokinin ratio is low?
- What precautions are taken during hardening?



Experiment 7

Aim

Indirect somatic embryogenesis in carrot (*Daucus carota*)

Key Terminology

- **Embryogenesis** Initiation or development of embryos from bipolar structures from somatic cells that parallel the development path of zygotic embryos. It may be either adventitious or *de novo* in origin.
- **Stages of embryogenesis** Distinct stages similar to globular shape, heart shape and torpedo shape; which the embryogenic cell passes through during embryogenesis.

Basic Principle

In somatic (asexual) embryogenesis, embryo-like structures, which develop into whole plants in a way similar to zygotic embryos, are formed from somatic tissues. These somatic embryos can be produced either directly or indirectly:

1. By inducing embryogenic cells within the preformed callus called—**organogenesis** (See Experiment 6; Chapter 4) and
2. Directly from pre-embryonic determined cells (i.e. without the production of intervening callus) which are ready to differentiate into embryoids called **somatic embryogenesis**.

Two nutritional media of different composition are required to obtain embryoids. In the first case the medium contains auxin to initiate embryogenic cells. Whereas, in the second case the medium either lacks auxin or has reduced level of auxin which is needed for subsequent development of the embryonic cells into embryoids and plantlets. In both the cases reduced level of nitrogen is required. Somatic embryogenesis usually proceeds in two distinct stages. In the initial stage, i.e. initiation of embryogenic cells, a high concentration of 2,4-D is

used. In the second stage, i.e. embryo production, embryo are produced in a medium without or with very low levels of 2,4-D. The embryogenic cells

pass through three different stages, e.g. **globular stage**, **heart stage** and **torpedo stage** to form embryoids (Fig. 4.7.1 and 4.7.2).

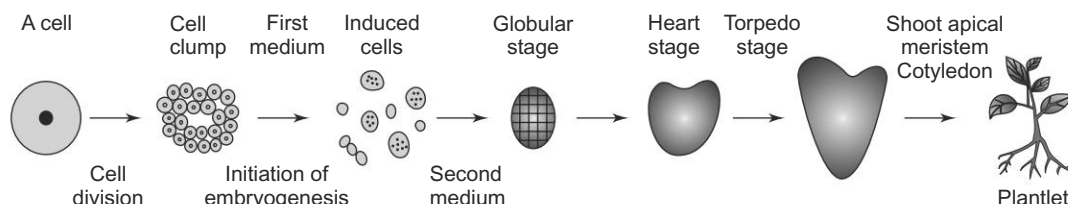


Fig. 4.7.1 Different stages of somatic embryogenesis (Diagramatic)

These embryoids can be separated and isolated mechanically by using glass beads. When the embryoids reach the torpedo stage, they are transferred to a filter paper bridge which is a sterile and plugged culture tube containing about 10 ml MS liquid medium supplemented with 0.2 ml/l of kinetin, sucrose or maltose (2% W/V) on which Whatman No. 1 filter paper is placed to make a bridge. Solid or liquid medium also contains Polyethylene glycol (PEG) and ascisic acid, on which embryoids develop into plantlets.

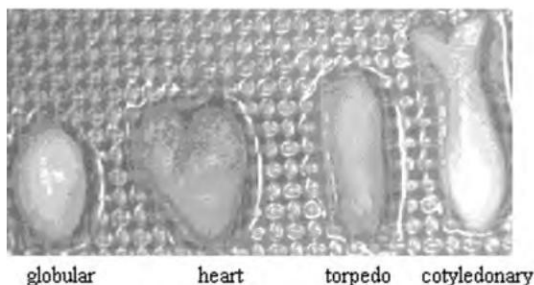


Fig. 4.7.2 Different stages of somatic embryogenesis (photo)

However, the major influence on tissue culture response appears to be genetic, with culture requirements varying between species and cultivars. Most of them respond poorly to culture *in vitro*. Many factors have been investigated for their ability to improve the culture response from elite cultivars, including media components (e.g. alternative carbon sources, macro- and microelement compositions), media preparation, method and donor plant condition and growth conditions. Some plants in which somatic embryogenesis has been

induced *in vitro* are—*Atropa belladonna*, *Brassica oleracea*, *Carica papaya*, *Coffea arabica*, *Citrus cinensis*, *Daucua carrota*, *Nicotiana tabacum*, *Pinus ponderosa* and *Saccharum officinarum*.

In this experiment we will learn to induce somatic embryogenesis from carrot root (*Daucus carrota*).

Materials

- MS Stock solutions (see Annexure IV)
- Plant material—fresh carrot roots
- Tween-20
- 0.1% Mercuric chloride, (HgCl₂)
- 1 mg/l of 2,4-D
- 0.025 mg/l Absciscic acid (ABA)
- Sterile forceps and sharp-blade (with holder)
- Sterile distilled water
- Sterile Whatman No.1 filter paper
- Culture-tubes and 100 ml wide-mouthed conical flasks
- Glass marker pencil

Methods

1. From preparation of carrot root explants to surface sterilization of cubes (follow Steps 1 to 4 as given in Experiment 5; Chapter 4).
2. For inducing callus from carrot root cubes, place the explants in culture tubes containing slants of MS basal media supplemented with 1 mg/l of 2,4-D.
3. This callus can be used to produce a cell-suspension by placing it in agitated wide-mouthed conical flasks containing liquid MS

- medium supplemented with 1 mg/l of 2,4-D. Agitated liquid medium are maintained for about 10 to 15 days.
- This cell suspension is maintained by repeated sub-culturing into 2,4-D containing medium for 2 or 3 times or until embryo develops to the globular stage.
 - After the third sub-culture or globular stage, remove the old 2,4-D containing medium and replace with fresh solid medium containing 0.025 mg/l of Absciscic acid. Continue till the production of embryoids or torpedo stage.
 - Transfer the embryoids on solid medium till complete plantlets are developed.
 - Acclimatize the plantlets using hardening materials.

Observations

Observe the different stages of somatic embryogenesis under microscope.

Precautions

- Observe closely during cell-suspension whether callus tissues are separated to single cells and thereafter keep watch of the development of globular, heart and torpedo stage.

Viva Questions



- Differentiate between organogenesis and embryogenesis.
- How will you prepare cell-suspension?
- What are the advantages of a liquid medium over a solid one?
- How does somatic embryogenesis differ from zygotic embryogenesis?



Experiment 8A

Aim *In vitro* germination of orchid seeds.

Key Terminology

- Naked embryo** Uncovered or unprotected embryo with endosperm, nucleus or other parts, generally observed in higher plants, e.g. dicots or monocots.
- Protocorm** Swelling or initiation of germination of naked embryo in orchids.
- Symbiotic germination** Association or requirement of other organism (e.g. fungus or bacterium) required for orchid embryo germination.

Basic Principles

Orchid family is the largest plant family and it comprises more than 25,000 species. Orchids are beautiful, mysterious plants which can be easily cultured in your home or lab using simple materials and little effort. Most of the orchids are used as costly cut flowers and some have medicinal values also. Different species are under constant pressure

which is threatening their existence. Scientists, orchidologists and orchid lovers are constantly trying to produce new hybrids and in search of mutants for their exotic beauty, exquisite flower colours, shapes, economy and grandeur.

Orchid seeds are naked embryo. In nature the rate of multiplication of orchid is very slow and hardly 3–5 per cent seeds germinate, which is negligible in comparison to indiscriminate removal and piracy of orchid plants. Plant tissue culture to some extent can help in this respect. Most of the orchid species produce fruit pods containing millions of naked seeds. In nature seeds **germinate symbiotically** in association with Mycorrhiza, whereas in the laboratory and home under sterile condition using orchid germinating medium it grows **asymbiotically**. Knudson's C (1947) basal medium is one of the most popular orchids germinating media.

In this experiment we will learn to germinate seeds of a terrestrial orchid, e.g. *Spathoglottis*

plicata (Bl.) which is commonly grown in pots in many north-eastern parts of India. This species is a fast growing orchid and from seedling to flowering stage it takes about 2½ to 3 years. The same procedure may be followed for germination of fruit pods of other orchid species or hybrids produced artificially.

Materials

- Mature fruit pods of *Spathoglottis plicata* (Bl.)
- Knudson's C basal media (See Annexure IV)
- 100 ml wide-mouthed conical flasks
- Absolute alcohol
- Spirit lamp
- Sterile surgical blade (with handle)
- 5 mg/l of NAA, 10 mg/l of IBA
- Glass-spreader
- Autoclave

Methods

1. At maturity fruit pods become yellowish along the septa, just few days before the pod splits or burst open and dehisces the powdery seeds. Therefore, pod should be used before splitting for inoculation of seeds. It is easier to use intact pods for inoculation. However, dehiscent seeds are also used for the same purpose, but need careful sterilization and handling.
2. Prepare one litre of Knudson's C basal medium (See Annexure IV), supplemented with 10 mg/l of IBA and 5 mg/l of NAA, which is added after membrane filtering and autoclaving at 121°C for 15 min and cooling down to 60°C.
3. Dispense (50 ml each) the sterile media in sterile 100 ml wide-mouthed conical flasks before laminar flow. Allow the flasks to solidify.
4. Clean and dress the fruit pod (remove all extra growths) and dip it in ethyl alcohol and flame it. Cut it into two equal halves and keep in a sterile petri-plate.
5. Take a flask, flame the mouth and open the plug. Take half of the pod and try to drop little powdery seeds into the flask by squeezing the pod. With the help of a sterile glass-spreader distribute the seeds throughout the surface of the media. Flame the plug and mouth of the flask and replace the plug.
6. Repeat the same process for all the flasks. Flasks are then incubated at $25 \pm 1^\circ\text{C}$ under dark in a tissue culture room.
7. After 4 weeks the naked orchid seeds swell and develop into round **protocorms** (Fig. 4.8.1). Prepare fresh media with same supplementation. The lumped protocorms are separated and uniformly distributed in the flasks. Now the flasks are exposed to 16 h photoperiod at 1,600 lux luminous intensity of light and 8 h dark period.
8. Leaves and roots will grow simultaneously. Sub-culture the plantlets after every 3 to 4 weeks of interval. When plantlets develop 4–5 leaves and roots are 2–5 cm long, remove the plantlets and wash the roots carefully. Spray the plantlets with fungicides and transfer them to pots.
9. Pots are filled with hardening composition (1:1 Charcoal granules : Leaf moulds, autoclaved) and leave them for acclimatization. Place the

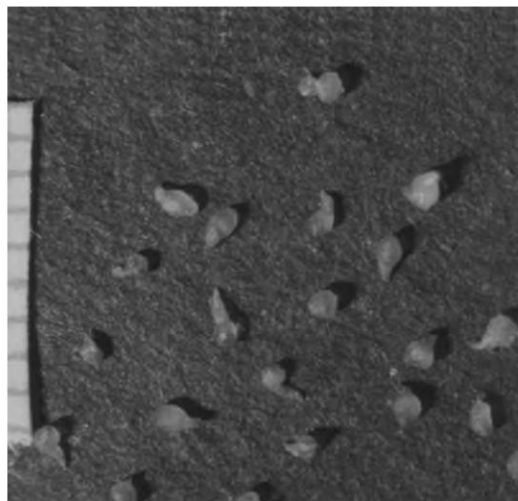


Fig. 4.8.1 Germination of orchid seeds and development of protocorms (each square = 1 sq.mm)

pots in shade having low temperature (around 26° to 28°C) and high humidity (80 to 85%). Spray intermittently with dilute Hogland or any other plant nutrient solutions. Reduce the frequency of spraying and increase the dilution gradually.

Observations

At the time of changing the media record period and length of leaf and root for finding the growth rate.

Viva Questions



- What are protocorms?
- Mention some importance of orchid?
- In India, where are orchids grow naturally?
- Orchid plants love which type of climate?
- Why orchid seeds are called naked embryo?
- Suggest an alternative sterilization method for orchid seeds for *in vitro* culture.



Experiment 8B

Aim

In vitro germination of Tobacco and Tomato seeds.

Key Terminology

- **Surfactant** It is a wetting agent that lowers the surface tension of a liquid, allowing easier spreading and lowers the interaction tension between two liquids.
- **Sterilization** The removal or destruction of all microorganisms including pathogenic and other bacteria, vegetative forms and spores from explant surfaces.
- **Disinfectant** An antimicrobial agent that is applied to living (seeds) or non-living objects to destroy microorganisms.
- **Sterilents** Compounds which destroy/kill bacteria, viruses or fungi.
- **Trichomes** Fine outgrowths or appendages on seed or plant surfaces.

Basic Principle

Viable seeds of monocot and dicot plants may be germinated *in vitro* for further culturing of shoot tip, leaf, stem or roots as an explant without any

Precautions

- If inoculated from intact fruit pod, there should not be any infection.
- Once the plug is removed from the conical flask, it should not be kept on the laminar flow table or any other place.
- Orchid seeds should be uniformly spread over the media for uniform growth of the plantlets.
- Rootless or abnormal or chlorophyll plantlets may be discarded at the time of sub-culture.

further sterilization. This makes *in vitro* culture of plants much easier. For such an *in vitro* culture simple MS basal medium (with or without IAA) may serve the purpose. However, it requires careful sterilization of the seed coat depending on its structure and seed coat thickness. As seed coat structure varies from seed to seed and it is permeable too. Therefore, concentration and duration of **sterilents**, viz. sodium hypochlorite, mercuric chloride, etc. will vary. In case the testa is hard and thick, removal of seed coat is essential or adopt techniques according to the requirement, e.g. for bigger seeds like tea and groundnut **surfactant** Twine-20 and **disinfectant** solution, 70% ethyl alcohol for 30 sec or 1 min may be used before using sodium hypochlorite.

In this experiment we will use tobacco and tomato seeds for *in vitro* culture.

Materials

- Fresh seeds of tobacco and tomato
- MS stock solutions (See Annexure IV)

- Petri-plates, 100 ml wide-mouthed conical flask
- Sodium hypochlorite 5% solution
- Ethyl alcohol 70%
- Dry sterile filter paper
- Sterile distilled water
- Vacuum pump
- Marking pencil

Methods

1. Wash the tobacco seeds thoroughly under running tap water and then imbibe them in 70% ethyl alcohol for 30 sec and then in 5% sodium hypochlorite for 5, 10 and 15 min (time of incubation may vary depending upon the seed coat structure or *in vitro* germination rate). Take out some seeds after 10 min, 15 min and 20 min.
2. Tomato seed coat contains hairy trichomes, which make the seeds to float in water. Therefore, take the seeds in a closed container fitted to a vacuum pump. Imbibe the seeds in 70% ethyl alcohol and then transfer the seeds in a container fitted to a vacuum pump. Run the pump for 30 min. Drain off the ethyl alcohol and replace with 5% sodium hypochlorite. Run the pump for 10, 15 and 20 min. After 10 min take out some seeds, again repeat it after 15 and 20 min.
3. After the surface sterilization, tobacco and tomato seeds should be thoroughly washed with sterile distilled water (4 or 5 times) to remove the sterilizing agent.
4. Remove the seeds from sterile distilled water and dry them in between dry sterile filter paper according to the duration of treatment.
5. Take sterilized MS basal medium in wide-mouthed conical flasks, mark the treatment number with a marker and uniformly spread the dried seeds on the medium according to the treatment number.
6. Incubate the flasks in dark for 8 to 10 days in the tissue culture room.

NOTE: Consult Experiments 3 and 6 Chapter 4.

Observations

Count the number of seeds germinated and record in the result table. Calculate percent of seeds germinated in tobacco and tomato under each treatment.

Results

Seeds	Sodium hypochlorite Treatment duration	Seeds inoculated	Seeds got infected	Seeds germinated	% of germination
Tobacco	0 min	30			
	5 min	30			
	10 min	30			
	15 min	30			
Tomato	0 min	30			
	10 min	30			
	15 min	30			
	20 min	30			

- (a) Percent (maximum) seed germination in tobacco under _____ min sodium hypochlorite treatment =
- (b) Percent (maximum) seed germination in tomato under _____ min sodium hypochlorite treatment =

Precautions

- Sterilization procedure must be performed under sterile conditions, i.e. in a laminar air-flow cabinet.
- Surface sterilization for longer duration may affect the viability of seeds.
- Duration of disinfectant may be increased/decreased based on germination result.

Viva Questions

- Suggest some other chemicals which can be used for surface sterilization in place of sodium hypochlorite.
- What is surface sterilization and how is it done?
- What technique you will apply for surface sterilization of rice, groundnut, pea, coriander, tea seeds?

**Experiment 9****Aim**

Extract protoplast from plant tissue and demonstrate the somatic cell fusion.

Key Terminology

- **Protoplasm** The material comprising the living part of a cell, including the cytoplasm, the nucleus and other organelles.
- **Somatic cell fusion** Amalgamation or to form a single entity of two protoplasm cells.
- **Electrofusion** Fusing of two cells with the help of electric current.
- **Fusogenic chemical** A chemical that helps or has a high potential to fuse protoplast membrane readily.
- **Osmo-regulator** Criteria or salt concentration that controls osmosis.
- **Osmoticum** Proper osmotic conditions (i.e. level of abscisic acid, potassium or high salt condition) that can maintain development *in vitro*.
- **Endosmosis** A process by which solvent molecules pass through a semi-permeable membrane from a less concentrated solution into a more concentrated one. Water moving inside the cell from outer source.
- **Exosmosis** Contrary to endosmosis, i.e. water moving out of the cell.

- **Homo-karyon** Fusion of two or more number of similar protoplast cells.
- **Hetero-karyon** Contrary to homo-karyon, i.e. fusion of two dissimilar protoplast cells.

Basic Principle

A plant cell consists of a protoplast protected by a rigid cell wall made up of cellulose as the main constituent. **Protoplast** (cell minus cell wall) is the biologically active and most significant material of cell. Many recent research works are based on “naked plant cell”. The effect of various metabolites, physical, chemical and biological environments on the cell functions can be monitored with the help of protoplasts. Plant cell wall consists of a mixture of **cellulose, hemicellulose, pectin, lignin, lipids, and protein**. Protoplasts can be isolated from various plant tissues, e.g. as leaves, flower petals, tissue like callus or cell suspension cultures. Cooking (1960) for the first time isolated the protoplasts of plant tissues by using cell wall degrading enzymes, viz. **cellulase, hemicellulase, pectinase and protease** extracted from a saprophytic fungus *Trichoderma viride* and cultured them *in vitro*.

Different cell wall digesting enzymes are used for preparation of protoplasts. The plant tissue

selected is cut into small pieces and is treated with a mixture known as **macerozyme**, which consists of pectinase, cellulase and hemicellulase. The enzyme pectinase, hydrolyzes the cementing material, which holds the adjacent cells together and the other two enzymes dissolve the cell wall leaving the cell or **plasma membrane** intact. The cell or plasma membrane protects the protoplasm, by encircling it, this is called **protoplast**. When a plant cell becomes a protoplast it loses its original shape and becomes rounded. Isolation of protoplast is carried out or maintained in a buffer which is usually supplemented with suitable **osmo-regulator**, e.g. **mannitol** or **sorbitol** to protect and maintain the protoplasts from osmotic potentials, i.e. checks the **endosmosis** or **exosmosis**, leading to the bursting or shrinking of the protoplasts. The optimum pH is maintained for facilitating the enzymatic digestion of the plant cell walls.

Protoplasts in a medium may undergo spontaneous fusion if they come in contact with one another. But fusion between protoplasts can be induced with the help of some (a) chemicals known as '**fusogens**' and (b) electric charge by a process which is termed as '**electrofusion**'.

(a) Fusogenic Chemicals It reduces the surface charges of the protoplasts and allows them to come closer to each other and result in their fusion. The most frequently used fusogenic chemical is polyethylene glycol (PEG). Other fusion inducing agents are the high pH, high Ca^{+} and sodium nitrate.

(b) Electrofusion A specially designed instrument used for bringing out protoplast fusion by high electrical charges.

Protoplast fusion is a helpful tool for:

- making crosses between plant species which are sexually not compatible,
- making crosses between two different genera—*intra* or *inter generic*
- transfer of nuclear and cytoplasmic genes.

The protoplast fusion may result in the formation of various types of fusion products:

- Homokaryon** – fusion products of two to many protoplasts from the same types of cell
- Heterokaryon**—fusion product of protoplasts isolated from two different tissues.

- Fusion products of protoplasts having colour differences (markers), e.g. protoplast containing chlorophyll and protoplast from leaf cells having anthocyanin pigmentation or flower petals.

In this experiment we will learn to isolate protoplasts and learn how to fuse two different coloured protoplasts to find out how it appears under the microscope.

Materials

- Plant tissues—coloured flower petals or young leaves with anthocyanin pigmentation and young green leaves or *in vitro* grown callus tissues, cell-suspension culture
- Macerozyme—Prepare 2% by mixing the appropriate quantities of the three enzymes, viz. pectinase, cellulase and hemicellulase
- Mannitol—in a plant tissue culture medium containing 0.6 M mannitol and no growth hormones, maintaining 5.8 pH
- Fusogen—Polyethylene glycol (PEG), 50% solution (dissolve 5 g in 10 ml of distilled water),
- Plant tissue culture media—MS Stock-solutions (*See Annexure IV*)
- Pipettes, Pasteur pipettes, pipetman and plastic pipette tips
- Sodium hypochlorite
- Sterile forceps
- Centrifuge
- Microscope
- Sterilized slides
- Membrane filter (0.45 μm mesh size)
- Nylon cloth (45 μm mesh size)

Methods

- Collect young and healthy leaves from plants of same species having green chlorophyll and anthocyanin pigmentation. Wash the leaves in running water and sterilize with sodium hypochlorite solution for 2 min and rinse the leaves for 6 min with sterile distilled water.
- For plasmolysis of cell, the lower epidermis covered by thin waxy cuticle is removed with forceps. Stripping should be done from midrib to margin of lamina. The stripped

surface of leaf is kept in mannitol solution (13% W/V) for 3 h to allow plasmolysis of cells.

- About 200 mg of leaves are taken and cut into pieces (from green and anthocyanin pigmented leaves) and then transferred to 2 ml macerozyme mixture already sterilized through a membrane filter (0.45 μm mesh). This facilitates the penetration of enzyme into tissues within 6 h at $25 \pm 2^\circ\text{C}$ incubation.
- The leaf debris are removed with forceps, and the enzyme solution containing protoplasts are filtered through a nylon mesh (45 μm). Keep the filtrate separate.
- The filtrate is centrifuged at 50 to 75 rpm for 5 min and the supernatant is decanted. Again a fresh MS medium plus 13% mannitol is added to the centrifuge. Repeated washing with nutrient medium, centrifugation and decantation are done (about thrice). Finally

the specific concentration of protoplast suspensions are prepared and kept in two separate tubes.

- Examine the two protoplasts under the microscope and observe the colours, shape and size. Take a clean and sterile slide. Put one drop of leaf protoplast from green pigmentation and one drop from anthocyanin pigmented leaf in the centre of the slide. Now add a drop of PEG along with the protoplast and keep it for 10 min. The PEG brings the two types of protoplasts very close side by side on the slide and helps in the fusion. Observe the slide under the microscope and note the changes in the fused cells.

Observations

Observe the following under the microscope after extraction and purification of protoplasts and after fusion:

Results

Record your observations in the table given below:

Characteristics of protoplast	After extraction of protoplast		After the fusion of protoplast	
	Green pigmentation	Anthocyanin pigmentation	Green pigmentation	Anthocyanin pigmentation
1. Pigmentation				
2. Size				
3. Shape				
4. Heterokaryon				
5. Homokaryon				

Precautions

- Prepare only the required amount of macerozyme and osmoticum.
- Protoplasts should not be agitated, handle carefully.

Viva Questions



- What is an osmoticum?
- What is a protoplast?
- How do protoplasts differ from protoplasm?
- What are the enzymes present in macerozyme?
- Describe the role of each enzyme in the removal of cell wall.
- What are the uses of isolated protoplasts?
- What are heterokaryon and homokaryon?
- Explain the role of PEG.

- Explain the role of mannitol.
- Explain the methods by which protoplasts can be induced to fuse.



Experiment 10

Aim Production of synthetic seeds and test their viability.

Key Terminology

- **Synthetic seeds** Artificially produced seeds with the help of chemicals, having enclosed embryo in endosperm and seed coat; have a small bead-like appearance.
- **Encapsulation** To enclose an embryo into an artificial substance.
- **Plant propagules** A plant part serving for propagation.
- **Alginate** A polysaccharide composed of glucuronic acid and mannuronic acid, and thus contains one carboxyl groups on each sugar moiety. In the presence of calcium or other multivalent cations, alginate forms a gel bead-like structure due to the formation of ionic bridges.

Basic Principle

The concept of artificial seeds was first introduced in 1970 as a noble analogue to plant seeds. The production of artificial seeds is especially useful for plants which do not produce viable seeds. It represents a method to propagate these plants. Artificial seeds are small sized and this provides further advantage to storage, handling, shipping and planting.

Artificial seeds are the living seed-like structures derived from somatic embryoids *in vitro* culture which is produced by **encapsulating** a plant propagule in a matrix and will allow it to grow into a plant. The preserved embryoids are also called **synthetic seeds**. Following are the three major parts of a seed (Fig. 4.10.1):

(i) Embryo The embryo is made up of one or two cotyledons attached to a central axis. The upper part of the axis contains a plumule at its tip. The plumule grows into the shoot system. The lower part of the axis consists of the hypocotyls and a radicle. The radicle grows into the root system.

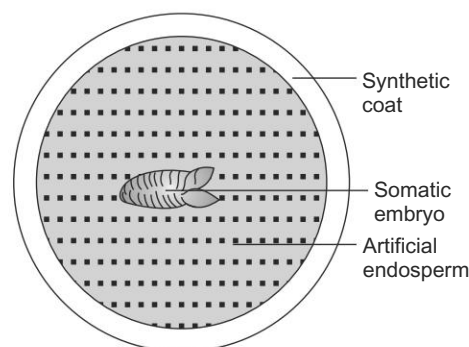


Fig. 4.10.1 Diagrammatic figure of an encapsulated artificial seed of plant

(ii) Endosperm Endosperm is the food reserve that the embryo uses during the early stages of germination. Before the embryo is able to produce its own food through photosynthesis, the endosperm provides vital nutrients to the embryo.

(iii) Testa The testa protects the embryo and endosperm from injury and drying out, it also makes sure that the embryo remains viable before germination. As germination occurs, water is absorbed and the seed coat breaks allowing the radicle to first emerge from the seed.

Plant **propagules** may consist of shoot buds or somatic embryos that have been grown aseptically in tissue culture. In culture, these plant propagules can easily grow into individual plants as we have the capacity to control its growth using chemicals provided in the culture media. These synthetic seeds can be stored for a longer period of time even up to 6 months without losing viability, especially when stored at 4°C.

In the production of artificial seeds, an artificial endosperm can be created within the encapsulating matrix. This can be done using various water-soluble **hydrogels** by alginate, gel

rite, carboxymethyl cellulose, made out of natural extracts from seed weeds (agar, carageenan or alginate), plants (Arabic or tragacanth), seed gums (guar locust bean, gum or tamarind) or microorganisms (dextran, gellan or xanthangum). These compounds will gel when mixed with or dropped into an appropriate electrolyte [CuSO_4 , $\text{Ca}(\text{NO}_3)_2$, CaCl_2 or NH_4Cl]. Ionic bonds are formed to produce stable complexes.

The major principle involved in the alginate encapsulation process is that the sodium alginate droplets containing the somatic embryo (Fig. 4.10.2) when dropped into the $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution form round and firm beads due to ion exchange between the Na^+ in sodium alginate with Ca^{2+} in the $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution. The hardness or rigidity of the capsule mainly depends upon the number of sodium ions exchanged with calcium ions. Hence, the concentration of the two gelling agents, i.e. sodium alginate and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and the complexing time should be standardized for the formation of the capsule with optimum bead hardness and rigidity. However, alginate based encapsulation was found to be the best. Alginate hydrogel is frequently selected as a matrix for synthetic seed because of its moderate viscosity and low spinnability of solution, low toxicity for somatic embryos and quick gellation, low cost and biocompatibility characteristics. Alginate is a straight chain, hydrophilic, colloidal polyuronic acid composed primarily of hydro- β -D-mannuronic acid residues with 1-4 linkages.



Fig. 4.10.2 Artificial or synthetic seeds produced by alginate encapsulation

In most cases a second coat covering the artificial endosperm is required to stimulate the

seed coat. The artificial seeds should be pliable enough to cushion and protect the embryo, yet allow germination and growth of the shoot bud or somatic embryo. It should be rigid enough to withstand rough handling during manufacture, transportation and planting. Incorporation of active charcoal improves the conversion and vigour of the encapsulated somatic embryos. It has been suggested that charcoal breaks up the alginate and thus increases respiration of somatic embryos (which otherwise lose vigour within a short period of storage). In addition, charcoal retains nutrients within the hydrogel capsule and slowly releases them to the growing embryo. Useful adjuvants such as nutrients, PGRs, pesticides, fungicides and antibiotics can be supplied to the plant propagule within the encapsulating matrix.

Potential Uses and Advantages of Synthetic Seeds

- (a) Can be preserved for long time at low temperature.
- (b) Rooting, hardening and conversion steps are waived off as these seeds can directly be sown in the field like natural seeds.
- (c) For the development of hybrid plants which have unstable genotype or show sterility in case that the seeds become sterile, the immature embryo from the seed can be rescued and then can be encapsulated artificially with appropriate growth medium to allow this maturation for germplasm conservation.
- (d) For various research and analysis purpose like studying the role of endosperm, etc.
- (e) To produce large number of the clones of elite species at a cheaper cost to supply adjuvant like plant growth regulators, pesticides, fungicides, etc.
- (f) Artificial seeds have the potential for providing an inexpensive plant delivering system. Process of planting can be easily mechanized and this allows the direct delivery of tissue cultured plant propagules to the field.
- (g) It also provides rapid bulking up for the production of individual genetically engineered plants.

- (h) Shoot buds cut from shoot cultures can be used for artificial seed production. They are trimmed to 2 to 3 mm in size and placed in encapsulated matrix. Somatic embryos formed from cultured plant parts are ideal for artificial seed production.
- (i) Moreover, orchid protocorms, white, friable callus may also be used for artificial seed production.

The lack of synchrony of somatic embryos is arguable, the single-most important hurdle to be overcome before advances leading to widespread commercialization of synthetic seeds can occur. Synchronized embryoid development is required for the efficient production of synthetic seeds.

This experiment can be an extension of Experiment Nos. 2, 6, 7 and 8 in this chapter. In this experiment we will produce synthetic seeds from meristemoids, embryoids, orchid protocorms, etc. available from the above experiments and their viability will be tested at an interval of 15 days.

Materials

- Plant materials—Rice meristemoids, carrot embryoids, orchid protocorms and carrot callus from Experiment Nos. 6, 7, 8 and 2, respectively may be used as a source of plant material for encapsulation,
- 2% to 4% Sodium alginate solution in MS basal media (See Annexure IV),
- Beakers 250 ml
- Calcium or sodium nitrate
- Adjuvants, e.g. PGRs, fungicides, pesticides, antibiotics, abscisic acid, etc.
- Pipette (with higher pore diameter than the usual size)
- Water soluble resins
- Sterile distilled water

Methods

Production of Somatic Embryogenesis

1. As an initial step, embryoids, meristemoids or orchid protocorms etc. are to be obtained from *in vitro* suspension culture, as a source of plant material for the production of encapsulated seeds.

2. In continuation of Experiment Nos. 6, 7 and 8, somatic embryos should be allowed to mature for using them in this experiment.

Encapsulation of Somatic Embryos There are generally two methods which may be used for encapsulation:

(a) Dropping Method

1. In this method somatic embryos are (plant materials from above Step 2) mixed with 2% to 4% sodium alginate solution prepared in MS-basal medium, supplemented with PGRs, sugar, extra nutrients, 1 μM of abscisic acid, and other adjuvants within the encapsulation matrix.
2. With the help of a pipette (with desirable pore diameter) drop the embryos in a bath of calcium salt, e.g. $\text{Ca}(\text{NO}_3)_2$ or $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution (75 to 100 mM) for 30 minutes. This results in very quick complex formation at the surface due to exchange of ions, i.e. Na^+ and Ca^{2+} . Consequently, individual embryos are enclosed into clear and hardened beads of about 4 mm diameter.
3. Rinse the gelled embryos with sterile distilled water and then sieve the beads through a nylon mesh; $\text{Ca}(\text{NO}_3)_2$ or $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution may be recycled. Beads may be stored at 4°C or used as synthetic seeds for immediate sowing.
4. As an additional coating beads are covered with a second coat (as per requirement) to stimulate the seed coat. For the artificial seeds to remain dormant until planting, a thin layer of water soluble resin is used to coat the encapsulation matrix.

(b) Moulding Method In this, the embryos are mixed with temperature-dependent gel-rite, where cells get gelled as the temperature is lowered. However, the synthetic seeds obtained by this method cannot be used directly into the field because they are very fragile and their nutrients will be attacked by the microflora present in the soil which will starve the embryo to death. As such it is not a popular method.

The beads or synthetic seeds produced are screened. Beads or synthetic seeds without any embryo should be discarded.

Test the Viability and Growth Vigour of Synthesized Seeds

Synthetic seeds produced by the above method are stored at 4°C. After every 15 days interval,

30–40 seeds are taken out and sown in the sand or soil amended with pesticides and fungicides. The number of seeds germinated is recorded.

Observations

Score the number of seeds germinated out of the total number of synthetic seeds sown and record in the table given below.

Results

Seeds sown after (in days)	No. of seeds sown	No. of seeds germinated	Germination percentage
15 days			
30 days			
45 days			

Precautions

- The shoot bud or somatic embryo is dropped into the complex ion solution and a capsule is formed and allowed to harden.
- Size of the capsule or bead is determined by the size of the shoot bud or somatic embryo
- and the inner diameter of the pipette used.
- Some of the aspects of the experiment need standardization, e.g. alginate concentration, pore diameter of pipette (for proper drop size) or $\text{Ca}(\text{NO}_3)_2$ or $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution concentration.

Viva Questions



- What is the composition of encapsulation matrix?
- What type of plant propagules can be used for production of artificial seeds?
- What are the advantages of artificial seeds?
- How do artificial seeds differ from natural seeds?
- What happens when sodium alginate and embryos are dropped into an appropriate electrolyte?

Notes and Sketches





5

Biochemistry, Enzyme and Fermentation Technology



Experiment 1A

Aim

Estimation of mouse or chicken liver glycogen by anthrone method.

Key Terminology

- **Glycogen** It is the most abundant polysaccharide for energy storage in animals.
- **Uridine diphosphate glucose (UDP-glucose)** During the synthesis of polysaccharides or disaccharides such as sucrose requires the glucose monomer to be in an activated form, which in case of glycogen and sucrose is called UDP-glucose.
- **GDP-glucose** Guanine-containing analog of UDP-glucose.

Basic Principle

Starch is the major molecule in which plants store energy, whereas glycogen is a carbohydrate reserve in animals, hence often referred to as '**animal starch**'. Glycogen is composed of units of glucose joined together by α (1 $\rightarrow$ 4) glycosidic bonds and α (1 $\rightarrow$ 6) glycosidic bonds at branching point. It is present in high concentrations in the liver, brain, etc. The anthrone reaction is the basis of rapid and convenient method for determination of hexoses, aldopentoses and hexauronic acid, either free or present in polysaccharide. The blue-green solution shows an absorption maximum at 630 nm, although some carbohydrate may give other colours. This method is non-stoichiometric and hence it is necessary to prepare a calibration curve using a

series of standards of known carbohydrate.

The synthesis of polysaccharides such as glycogen, cellulose, and starch or disaccharides such as sucrose requires the glucose monomer to be in activated form, which in the case of glycogen and sucrose is called uridine diphosphate glucose (**UDP-glucose**). The enzyme **glycogen synthetase** catalyzes the transfer of glucose from UDP-glucose to the terminal hydroxyl group of a growing glycogen strand. Biosynthesis of cellulose is thought to involve **GDP-glucose** (the guanine-containing analog of UDP-glucose) or other nucleotide diphosphate sugar (depending on the species of plant), but the details of the process are not clear. Starch synthesis occurs when glyceraldehydes 3-phosphate is converted to glucose 1-phosphate, which in turn is used to synthesize the immediate activated precursor of starch, a nucleoside diphosphate sugar called adenosine diphosphate glucose (**ADP-glucose**). Starch is stored as large grains in the stroma during periods of excess photosynthetic activity and is broken down at night to supply the energy needs of the plant. However, much of the glyceraldehydes 3-phosphate produced in chloroplasts is transported to the cytoplasm, where it is converted into glucose 1-phosphate and fructose 6-phosphate by reversal

of some steps in glycolysis. Glucose 1-phosphate becomes converted to UDP-glucose, which combines with fructose 6-phosphate to form sucrose phosphate, the immediate precursor of sucrose.

Materials

- Standard glucose
- Anthrone reagent
- Conc. H_2SO_4
- Potassium hydroxide
- Na_2SO_4
- Ethanol
- Distilled water
- Measuring cylinders
- Storage bottles
- Pipettes
- Test-tubes
- Beaker
- Volumetric flask
- Falcon tubes
- Electronic balance

- Spectrophotometer and Cuvettes
- Heater
- Refrigerated centrifuge
- Specimen/Sample: Mouse or chicken liver.

Methods

Preparation of Calibration Curve of Glucose

1. Take different concentrations of standard glucose solution in different test-tubes as shown in Table 1. Also keep a blank (i.e. do not add any standard glucose solution).
2. Adjust the volume to 1 ml with distilled water.
3. Add 5 ml of 2% anthrone reagent to each tube and mix rapidly by swirling.
4. Keep the tubes in a preheated boiling water bath for 10 minutes and then cool.
5. Measure the absorbance (OD) of each standard solution using a spectrophotometer at a wavelength 630 nm.

Observation

Table 5.1.1 Showing different concentrations of glucose, distilled water and Anthrone reagents to be mixed for standard graph.

SL.No.	GLUCOSE		$d\text{H}_2\text{O}$ (ml)	ANTHRONE REAGENT (ml)	TOTAL VOLUME (ml)	OD AT 630 nm
	μg	ml				
Blank	0	0.0	1.0	5	6	0.00
1	20	0.2	0.8	5	6	
2	40	0.4	0.6	5	6	
3	60	0.6	0.4	5	6	
4	80	0.8	0.2	5	6	
5	100	1.0	0.0	5	6	

Now, plot a graph on a graph paper taking optical density on Y-axis and the known glucose concentration on X-axis. The graph obtained should be a straight line and is the standard or calibrated curve of glucose.

Note: After preparation of the standard curve, perform the estimation of glycogen from any given sample (e.g. mouse) following the protocol provided in the next experiment (Experiment 1B).



Experiment 1B

Aim Preparation of liver homogenate and estimation of liver glycogen.

Methods

1. Weigh 5 mg of liver and immediately transfer to boiling 30% KOH solution, and keep in a boiling water bath for 15 min.
2. To the above mixture add 1.2 ml volume of 95% ethanol with 0.5 ml saturated sodium sulphate (Na_2SO_4) to precipitate glycogen by allowing the mixture to stand overnight.
3. Next day, centrifuge the overnight mixture at 3000 rpm for 10 min.
4. Decant and discard the supernatant, and then wash the residual centrifuged glycogen twice with 65% ethanol. Retain the residue by centrifugation and decant the alcohol out.
5. Dissolve the residue in distilled water and transfer to a 100 ml volumetric flask and adjust the volume up to 100 ml.
6. Transfer 10 ml of the solution to another 100 ml volumetric flask and adjust the volume up to 100 ml.
7. Pipette 5 ml of 2% anthrone solution into 4 test-tubes and cool by keeping in ice water.
8. Pipette 1 ml of the glycogen solution and layer it on the top of the acid solution, and mix it rapidly by swirling.
9. Stop the reaction by rapidly boiling in a boiling water bath for 10 min. Cool the tubes by holding it under a running water system and the green colour obtained should be read against sulphuric acid in a spectrophotometer at 630 nm.
10. The amount of glycogen should be estimated from the standard/calibration curve as μg of glycogen per ml and finally as mg glucose per gm weight of liver tissue.

Observations

Amount of test sample	Anthrone reagent	Total volume	O.D. ₆₃₀
1 ml	5 ml	6 ml	

The OD of the test sample thus obtained should be plotted on the calibration/standard curve of glucose and from that amount of the glucose ($\mu\text{g}/\text{mg}$ liver) present in the mouse liver should be calculated.

Calculations

From the standard curve graph, it was found that the OD of the test sample (glycogen from the mouse or chicken liver) corresponds to a concentration of $\dots X \dots \mu\text{g}/\text{ml}$ of reaction mixture.

Since,

1 ml of test sample is equivalent to $X \mu\text{g}$ glucose (from standard curve)

So, 100 ml of test sample is equivalent to $100 \times X \mu\text{g}$ glucose

Therefore, 10 ml of test sample contains $100X \mu\text{g}$ glucose

Hence, 100 ml of liver extract contains $10 \times 100X = 1000X \mu\text{g}$ glucose

Since, 5 mg of liver sample is present in 100 ml of liver extract

Therefore, 5 mg of mouse or chicken liver contains $1000X \mu\text{g}$ glucose

Hence, 1 mg of mouse or chicken liver contains $1000X/5 = Y \mu\text{g}$ glucose

Result

Total amount of glycogen present in mouse or chicken liver is expressed as $Y \mu\text{g}$ of glucose/mg liver.

Precautions

- The anthrone reaction is not suitable when protein containing large amount of tryptophan are present, since a red colour is formed under

these conditions. The extinction coefficient depends upon the compound investigated.

- One should be extremely careful while handling sulphuric acid.

Viva Questions

- What is the form of stored carbohydrate macro molecular energy in plants and animals?
- What are the four most prevalent polysaccharides in eukaryotes?
- What is the composition of starch and glycogen? How are they digested?
- Compare the structure of glycogen and starch.
- How are polysaccharides synthesized in animals?

**Experiment 2****Aim**

Extraction of lipid from egg yolk.

Key Terminology

- **Lipids** Water-insoluble, greasy or oily substances present in cells or food products.
- **Lipidomics** Study of complete lipid profile of a cell or cellular system.
- **Thin layer chromatography (TLC)** is a technique used to separate biological components with the help of a moving solvent on an absorbent surface.

Basic Principle

Lipids are water-insoluble greasy substances that are extractable from cells and tissues. The most abundant kind of lipids are **non-polar neutral lipid** or fat, i.e. the **triglycerols**, etc. Lipids can be extracted using a mixture of polar and non-polar solvents like chloroform and methanol. The effectiveness of procedure, to a large extent, depends on the chemical nature of the lipid component and the kind of complex or association in which they occur in the cell. There are mainly five types of association, viz. van der-Waals forces, hydrophobic associations, hydrogen bonding, electrostatic and covalent bonding.

Hydrophobically associated lipids are separated with relatively non-polar solvents like chloroform. But membrane-associated lipids require polar solvents like methanol to disrupt the **hydrogen bonding** or **electrostatic lipids** cannot be extracted di-

rectly by any solvent but must first be cleared from the complex by acid and alkaline hydrolysis.

Lipids of Egg Yolk Egg yolk is a rich source of lipids. It contains both neutral lipids, i.e. triglycerides and polar phospholipids such as phosphatidylcholine, phosphatidyl ethanol amine, phosphatidyl serine and phosphatidyl inositol. Since both polar and non-polar lipids are present, for extraction of lipids from egg yolks, a mixture of polar and non-polar solvent, viz., chloroform and methanol (2:1 v/v) can be used.

Materials

- Separating funnel
- Whatmann filter paper No. 1
- Chloroform-methanol (2:1 v/v)
- NaCl
- Beaker
- Digital balance
- Specimen: 1 egg yolk

Methods

1. Take a pre-cleaned beaker and weigh.
2. Take the egg, and break carefully, retaining the yolk portion only in the beaker. Weigh the beaker again with the yolk.
3. Add 50 ml of mixture of chloroform-methanol (2:1 v/v) solvent to the yolk and

shake vigorously for 15 min, resulting in the formation of a white precipitate which floats up to the surface and a deep yellowish liquid which collects at the bottom.

4. Place a Whatmann filter paper No. 1 on a separating funnel, and filter the mixture through it.
5. Collect the filtrate and treat with 1/5 of the volume of 0.9% NaCl solution. Shake the mixture vigorously for 10 min and store overnight in a refrigerator.

Observation

Two distinct layers are formed. The top one contains water-soluble impurities extracted by NaCl while the lower layer contains a solution of

lipids. Take out the clean lower layer carefully and measure the volume.

Results

The volume of lipid content was measured to be ... ml from ... mg of egg yolk.

Precautions

- Both chloroform and methanol are volatile, and therefore should be prepared freshly and adequately.
- Keep your face away when using chloroform.
- Keep the mixture away from fire.
- The refrigeration should be maintained at 4°C.

Viva Questions



- What distinguishes lipids from carbohydrates, proteins and nucleic acids?
- What are the two most important functions that lipids perform in living organisms?
- What are the three major kinds of membrane lipids?
- What factors constitute to the fluidity of lipid membranes?
- How are macro-molecular lipid membranes synthesized?



Experiment 3

Aim

Separate lipids by thin layer chromatography (TLC) method.

Key Terminology

- **Fractionation** A process that uses heat to separate a substance into its components.
- **Adsorption** The accumulation of atoms or molecules of a gas to form a thin film on the surface of a solid or material.
- **Silica gel** It is a granular, vitreous, highly porous form of silica made synthetically from sodium silicate.

Basic Principle

Lipids in biological material, present as a complex and are first fractioned into a member of groups by solvent extraction. Resolution of the compounds within each group can then be done by **thin layer chromatography (TLC)**. The solvent usually is selected according to the lipids to be screened. In general terms, neutral lipids are separated with non-

polar solvents and charged lipids with polar solvents. A number of supporting media may be implemented for lipid **fractionation**, but generally the actual choice of absorbent depends on the groups of lipids and solvent employed. Silica gel has been widely used and separation with this material is on the basis of **adsorption** and partition.

Materials

- Glass plates
- Solvent (chloroform : Methanol : water:: 65:25:10)
- Silica gel
- Lipid extracted from egg yolk

Methods

1. Prepare a slurry of silica gel in water.
2. Apply the slurry on the surface of the glass plate uniformly using a clean glass rod.

3. Leave the plates to dry for 2 h.
4. The plates are activated by heating in an oven at 110°C for 30 min.
5. Allow the plates to cool down at room temperature.
6. When cold, the solution of lipid extracted from egg yolk should be carefully spotted on the plate along the margin.
7. Place the spotted plate in the separating chamber with the lower margin dipped in the solvent. The chamber should be vapour saturated by sealing the chamber with a glass plate to prevent entry of air.
8. Allow the separation to take place for 30 min.

Detection Method

1. Remove the plates from the separation chamber and allow to dry. Mark the solvent front.
2. After drying, place the plates in iodine chamber for localization of the lipids. Distinct brown colour spots appear at place where the lipids have separated (Fig. 5.3.1).
3. Take out the plates from the iodine chamber and quickly mark out the position of the lipids.

Observation

The lipids are separated into two distinct brown spots on the TLC plates. Detection and identifica-

Viva Questions • Same as in Experiment 3.



Experiment 4

Aim Protein extraction and estimation from mouse liver.

Key Terminology

- **Proteome** Total protein content or representation of a cell.
- **Proteases** Protein degrading or eating enzymes.
- **Protease inhibitors** Chemicals that can inhibit the action of the proteases if at all present during the protein isolation etc.

tion of the types of lipid present in the samples requires further chemical treatment and comparison with standard chromatogram.

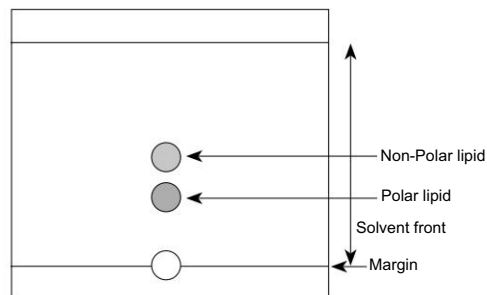


Fig. 5.3.1 Typical TLC plate showing different types of lipids

Results

The lipid extracted from egg yolk could be separated into a non-polar and polar lipid part successfully using the thin layer chromatography technique.

Precautions

- The glass plates should be grease free.
- The solvent should be made fresh and pre-incubated in the glass chamber before transferring the silica plates into it.

- **Lysis buffer** A buffer used for the purpose of lysing cells for use in experiments that analyze the compounds of the cells (e.g. Western Blot).

Basic Principle

The total protein content inside the cell is extracted out by lysing the cells or cell membranes using a

suitable **lysis buffer**, and stabilizing the protein by safeguarding against external proteases. The cell debris are precipitated out by centrifugation and the protein content in the supernatant part is quantified and used for further investigations.

Materials

- Magnesium containing lysis buffer (MLB buffer) (see *Annexure II, No. 3*).
- Add 10 µg/ml Leupeptin, 10 µg/ml Aprotinin, 5 µg/ml PMSF as protease inhibitors just before use.
- Specimen: 2 cm³ of mouse/chicken liver sample.
- Ice
- Centrifuge
- Homogenizer
- Rocker
- Micropipettes
- Eppendorf tubes
- Autoclaved tips
- Falcon tubes

Methods

1. Measure out a 2 cm³ of freshly isolated or properly stored liver sample dissected out of a mouse/chicken.
2. Put the liver piece in a 2 ml eppendorf tube or 15 ml falcon tubes. Add 1 ml of MLB buffer to the sample and homogenize using a mechanical homogenizer. (Alternatively, the

tissue may be crushed in a mortar and pestle by adding liquid nitrogen, and then add and mix in MLB lysis buffer).

3. Put the homogenized sample on ice for 5 minutes and then place in a rotating rocker in a cold room/cold condition for at least 20–30 min.
4. Centrifuge the tubes at 10,000 rpm for 5 mins at 4°C to precipitate out the cellular debris.
5. Collect the supernatant containing the protein in fresh eppendorf tubes and store at –20°C; or alternatively quantify the protein by Folin Lowry method (See *Experiment 5*).

Precautions

- Proteins are easily degradable and therefore utmost care should be taken to save proteins from the external proteases.
- The protease inhibitors should be added freshly before extraction as the half life of most of the protease inhibitors is very less.
- Adequate amount of MLB buffer should be used, so that the tissue is completely dipped in the extraction buffer when performing the homogenization.
- The extracted proteins should be stored at –20°C, and should be thawed on ice if and whenever required, and as less as possible, as too much of thawing and freezing results in the degradation of protein concentration.

Viva Questions



- What are proteins and what general features do they share in the living cell?
- What are “essential” amino acids?
- How are amino acids linked together to form proteins by the living cells?
- What is the difference between a polypeptide chain and a protein?
- How can electrophoresis be used to study protein?



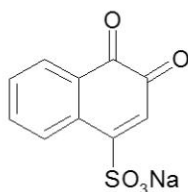
Experiment 5

Aim

Preparation of standard curve of protein (BSA) by Folin-Lowry method and determination of unknown protein concentration.

Key Terminology

- **BSA** Bovine serum albumin is standard purified commercially available protein extracted from bovines.
- **Folin-Ciocaltean reagent:** Folin's reagent or sodium 1, 2-naphthoquinone-4-sulfonate is a chemical reagent used to measure levels of amines and amino acids.



Basic Principle

Protein reacts with the Folin-Ciocaltean reagent to give a coloured complex. The colour so formed is due to the reaction of the alkaline copper with the protein as in the burette test and reduction of phosphomolybdate by tyrosine and tryptophane present in the protein. The intensity of the colour depends on the amount of the aromatic acids present and thus will vary for different proteins.

The burette reagent consists of a solution of copper sulphate (CuSO_4) in an alkaline solution of sodium potassium tartarate. On reacting with a protein the Cu^{++} ions co-ordinate with the peptide bonds of the protein and get reduced to Cu^+ ions leading to the formation of a purple-violet colouration.

Materials

- Sodium carbonate
- NaOH
- Copper sulphate (CuSO_4)
- Sodium potassium tartarate
- Folin-Ciocaltean reagent (Commercially available, e.g. Merck)
- Bovine serum albumin (BSA)
- Distilled water
- Test-tubes
- Conical flask
- Beaker
- Measuring cylinders
- Micropipette
- Spectrophotometer and cuvettes
- Shaker
- Electronic balance

Chemical Preparation

Solution 1: *Alkaline sodium carbonate solution:*

Dissolve 2 g sodium carbonate in 100 ml of 0.1N NaOH solution.

Solution 2: *Copper sulphate potassium tartarate*

solution (10 ml): Dissolve 0.05 g copper sulphate in 10 ml of 1% sodium potassium tartarate solution.

Alkali reagent: Prepare the alkali reagent by adding 100 ml of solution 1 and 2 ml of solution 2.

Standard protein (BSA): Prepare it fresh in a concentration of 1 mg/ml.

Folin-Ciocaltean reagent: Dilute in the ratio of 1:1 with distilled water.

Methods

1. Prepare different dilutions of standard protein solution (BSA) in different test-tubes (e.g. 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml adjusted up to 1 ml with addition of distilled water). Always keep a blank (without standard protein).
2. Add 5 ml of alkaline reagent in the tubes, mix, and keep at room temperature for 10 min.
3. Add 0.5 ml of (1:1) diluted Folin-Ciocaltean reagent and mix immediately. Allow the reaction mix to stand for 30 min.
4. After 30 min, measure the OD at 660 nm against the reagent blank using the spectrophotometer.
5. Plot the standard curve based on the OD values (Y-axis) v/s the concentration of BSA (X-axis) using a standard graph paper.
6. For unknown samples, take 1 μl of the unknown samples and adjust the volume up to 1 ml by addition of distilled water. Repeat steps 2 and 4.
7. Plot the OD for the unknown sample on the standard curve and calculate the protein concentration.

Observation**Table 5.5.1** Spectrophotometric observation for standard BSA concentration

SERIAL No.	BSA (STANDARD PROTEIN) 1MG/ML	DISTILLED WATER (ML)	ALKALI REAGENT (ML)	FOLIN-CIOCALTEAN REAGENT (ML)	OD AT 660 NM
Blank	0.0	1	5	0.5	
1	0.2	0.8	5	0.5	
2	0.4	0.6	5	0.5	
3	0.6	0.4	5	0.5	
4	0.8	0.2	5	0.5	
5	1.0	0.0	5	0.5	

Now, plot the graph based on the OD for specific BSA concentration which should be a straight line.

Calculate the concentration of the unknown protein samples by plotting the OD₆₆₀ and mapping for the corresponding concentration based on the standard curve.

Unknown samples:

Samples	Optical density	Concentration (µg/ml)
Unknown 1		
Unknown 2		

Result

From the above experiment and standard curve it has been found that a straight line is obtained

having positive slope when OD values taken at 660 nm and plotted on a graph paper.

The OD values of the unknown proteins were plotted against concentration of BSA on the standard curve and the concentrations of the unknown samples were determined which were found to be:

Unknown 1 = ... µg/ml

Unknown 2 = ... µg/ml

Precautions

- Store BSA at 4°C and always use fresh BSA solution for plotting the standard graph.
- Measure the OD of unknown sample against the blank for several times, till two observations coincide.

Viva Questions

- If a protein other than BSA is used, will the values of absorbance differ for the standard?
- How does the Folin-Lowry method works?
- If the protein in the unknown test sample is higher than 10 mg/ml (beyond the detection range of the BSA protein content) how will you estimate protein in this sample?

**Experiment 6****Aim**

To study the effect of the enzyme lactase on milk.

Key Terminology

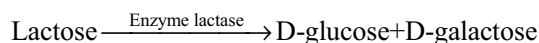
- **Enzyme** A bio-catalyst produced by living cells.

- **Disaccharide** Six-sided sugar, e.g. glucose or galactose.
- **Hydrolysis** Addition of water molecule.

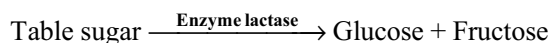
- **Skim milk** Milk from which cream has been skimmed or removed.
- **Denaturing enzyme** When an enzyme is exposed to extreme conditions such as high temperature or unsuitable pH, the active sites are broken down.

Basic Principle

Lactose, the sugar found in milk, is a **disaccharide** composed of glucose and galactose (both six-sided sugar). Ordinary table sugar, sucrose, is also a disaccharide composed of fructose (five-sided sugar) and glucose. The enzyme lactase breaks down lactose into galactose and glucose. Although lactose is similar to sucrose, lactase will break down only lactose because of the shape of the sugar. The specificity of enzyme reaction of breaking down of sucrose and lactose is based on shape. When hydrolyzed by the enzyme lactase, lactose yields equimolar quantities of D-glucose and D-galactose.



Similarly, when the enzyme lactase hydrolyses Table-sugar, it yields glucose and fructose.



In this experiment, we will study the effect of the enzyme lactase breaking lactose into galactose and glucose. We will also observe what happens if the shape of lactose is changed due to heating.

Materials

- Lactase tablets (may be purchased from a medical store)
- 15 ml of milk (cow or any milk)
- Sucrose 5 g
- 10 and 100 ml measuring cylinder
- A 500 ml beaker (Borosil)
- Test-tubes (Borosil) with stand
- Distilled water
- Glass marking pencil
- Stop watch
- Hot plate or burner (for heating)
- Glucose test strips (available in medical stores, used by diabetic patients)
- Glass stirring rod

Methods

Preparation of Solutions Keep following solutions ready before conducting the experiment:

1. *Enzyme solution*—Add lactase tablet to 200 ml of distilled water. Stir until the tablet has dissolved.
2. *Skim milk*—milk from which cream has been removed.
3. *Sucrose solution*—Add 5 g of table sugar to 100 ml of distilled water. Stir to dissolve the sugar.
4. *Denatured enzyme solution*—
 - (a) Place 20 ml of enzyme solution into a test-tube,
 - (b) Add 200 ml of water to a 500 ml beaker,
 - (c) Place the test-tube in the beaker,
 - (d) Place the beaker along with test-tube on a hot plate or burner,
 - (e) Boil the water in the beaker for 30 min. Cool it down to room temperature.

Procedure After all solutions are ready follow the following steps:

1. Label the test-tubes as—
 - #A. Test-tube with skim milk + Enzyme solution
 - #B. Test-tube with skim milk + Water
 - #C. Test-tube with milk + Denatured enzyme solution
 - #D. Test-tube with sucrose solution + Enzyme solution
 - #E. Test-tube sucrose solution + Water
2. *In test-tube A:* Add 2 ml of skim milk and 1 ml of enzyme solution.
3. Wait for 5 min (or more) and test for glucose with the glucose test strip. Record your data in the result table.
4. *In test-tube B:* Add 2 ml of skim milk and 1 ml of water and repeat Step 3.
5. *In test-tube C:* Add 2 ml of skim milk and 1 ml of denatured enzyme solution and repeat Step 3.
6. *In test-tube D:* Add 2 ml of the sucrose solution and 1 ml of enzyme solution. Repeat Step 3.
7. *In test-tube E:* Add 2 ml of the sucrose solution and 1 ml of water. Repeat Step 3.

Observations

Find out presence and absence of glucose in the test-tube.

Results

Indicate the presence or absence of glucose in the solutions by '+' and '-' mark, respectively.

Type of solution	Glucose result	
	Positive '+'	Negative '-'
Test-tube A: Skim milk + Enzyme		
Test-tube B: Skim milk + Water		
Test-tube C: Skim milk + Denatured enzyme sol.		
Test-tube D: Sucrose sol.+ Enzyme sol.		
Test-tube E: Sucrose sol.+ Water		

Precautions

- Denaturing the enzyme is very difficult. Boil for longer period of time.

Viva Questions

- Why did the enzyme react to lactose but not to sucrose?
- What happened when the enzyme was boiled?
- What type of reaction occurred when it was heated?
- Would lowering the pH of the enzyme solution affect the enzyme? Why or why not?

**Experiment 7****Aim**

To immobilize yeast cells by sodium alginate entrapment method and to study the rate of conversion of glucose by immobilized yeast cells.

Key Terminology

- **Immobilization** It is the formation of more organic product, which is resistant to break down.
- **Carrier matrices** The enzyme molecule to a distinct phase from the one in which the substrate and the products are present. The materials covalent binding (of enzyme) used for immobilization of enzymes, called carrier matrices, are usually inert polymers or inorganic materials.
- **Covalent bonding (of enzyme)** A chemical bond formed by the sharing of one or more electrons, especially pairs of electrons, between atoms.

Basic Principle

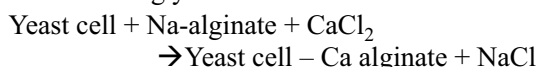
Background Immobilization can be defined as confining the cells or molecules to a distinct phase from the one in which the substrates and the products are present. This may be achieved by fixing the cell or the molecule to or within some suitable material. It is critical that the substrates and the product move freely in and out of the phase to which the enzyme molecules are confined. Immobilization of a cell or molecule does not necessarily render them immobile.

The materials used for immobilization of enzymes called **carrier matrices** are usually inert polymers or inorganic materials. The ideal carrier matrices have the following properties.

- (i) Low cost
- (ii) Inertness
- (iii) Physical strength
- (iv) Stability
- (v) Regenerability after the useful lifetime of immobilized cell, molecule in enzyme.
- (vi) Enhancement of enzyme specificity
- (vii) Reduction in product inhibition
- (viii) A shift in the pH optimum for enzyme action to the desired value for the process
- (ix) Reduction in microbial contamination and nonspecific adsorption.

There are various types of immobilization methods. These include (i) **adsorption** (ii) **covalent bonding** (iii) **entrapment** and (iv) **membrane confinement**. In the entrapment method, the cell or the molecule are held or entrapped within suitable gels or fibres and there may not be covalent bond formation between the cell and the matrix. A non-covalent entrapment may be viewed as putting the cell in a molecular cage just as a caged bird/animal.

When yeast cell suspension is mixed with sodium-alginate, it forms a gel and this gel when put drop-wise in CaCl_2 contained in a beaker above the magnetic stirrer produces spherical gel beads containing yeast cell inside.



The yeast cells after immobilization retain their property of utilizing glucose in the solution. So the **amount of glucose utilized by the yeast cell is estimated using Benedict's reagent at regular intervals of time**. Glucose in the unknown solution can be calculated using the formula: 1 ml of Benedict's reagent (Br) = 2 mg of glucose.

Materials

- Yeast cells
- Calcium chloride
- Sodium alginate
- Benedict's reagent
- Sodium carbonate
- Test-tubes
- Pipettes
- Magnetic cylinder

- Magnetic stirrer
- Spirit lamp
- Distilled water

Preparation of Reagents:

- (i) *CaCl₂ solution*: 1.47 g of CaCl_2 was dissolved in 100 ml of distilled water.
- (ii) *2% Na alginate solution*: 2 g of Na-alginate was dissolved in 100 ml of distilled water.

Methods

Immobilized Yeast Cell Preparation

1. Weigh 100 mg of yeast cells and mix with 230 ml of distilled water. Add 4.6 g of Na-alginate to this solution to make 2% Na-alginate solution (warm a little to dissolve, if needed).
2. Take a dropper or a 5 ml pipette and suck the suspension of yeast-Na-alginate solution in and allow to fall drop by drop to CaCl_2 solution placed in a beaker on a magnetic stirrer. The drops soon form beads which should be left at room temperature for about an hour.
3. Wash the beads with distilled water and store at room temperature in water.

Estimation of Glucose Utilization by Yeast

Cell Take the beads in a 250 ml beaker containing 100 ml of 2% glucose solution. The utilization of glucose by immobilized yeast cell needs to be estimated at regular intervals using the following steps:

1. Take 2 ml of Benedict's reagent in a test-tube and add 240 mg of Na_2CO_3 to it.
2. Heat the solution up to its boiling point using a spirit lamp/burner.
3. When the solution starts to boil, take out 2 ml of glucose from the immobilized medium with the help of the pipette and add this unknown glucose solutions drop-wise and titrate against the hot Benedict's reagents solution. At the end point the blue colour of Benedict's reagent disappear completely and the solution becomes colourless.
4. Carefully note the volume of the unknown glucose solution added.

5. The glucose in the unknown solution should be calculated using the formula:
1 ml of Benedict's reagent = 2 mg of glucose
6. Tabulate the results and plot a graph for the reduction of glucose against the time taken.

Observation

The results obtained for the experiment are tabulated as follows:

Residual glucose concentration (2%) at different intervals due to transformation of glucose by immobilized yeast cells.

Sl. No	Time (min)	Volume of glucose (ml)	Concentration of glucose (mg/ml)	Reduction % of glucose
1				
2				
3				
4				
5				
6				

Results

The yeast cell retains its property of utilizing glucose after immobilization.% of substrate is converted in min. Therefore, immobilized yeast cell can be used to produce products like ethanol.

Precautions

- Yeast Na-alginate solution drops should be allowed to fall at an equal interval.

Viva Questions

- What is immobilization of an enzyme?
- What are the various immobilization methods?
- What is the role of yeast cells? What happens when it is added to Na-alginate solution?
- Can the utilized yeast cells be reused?
- What is covalent bonding of an enzyme?

**Experiment 8**

Aim Protease estimation from fruits.

Key Terminology

- **Suspended particles of proteins** A method for removing suspended particles from a soluble protein solution comprising the steps of

filtering the soluble protein solution.

- **Denaturation** a process in which proteins or nucleic acids lose their tertiary structure and secondary structure by application of some external stress.

- **Colloidal** A colloidal system consists of two separate phases: a dispersed phase (or internal phase) and a continuous phase (or dispersion medium).

Basic Principle

Background In certain fruits, such as pineapple and mango, the flesh contains **protein-digesting enzymes (proteases)**. These may play a part in helping to soften the fruit tissues as the fruit ripens. Applications of plant proteases include use in the pharmaceutical industry as a blood anti-coagulant, and in the prevention of **pertinacious hazes in chill-proof beers**.

Protease enzymes are added to a milky colloidal suspension of egg albumen. As the protease enzymes digest the suspended particles of proteins, the mixture becomes more transparent. The absorbance changes in the reaction mixture are measured with a colorimeter. The protease content, for example in extracts of fruits, can be assayed by measuring the rate at which the solution of egg albumen and extract becomes clearer.

Materials

- Egg albumen
- A stop watch
- Fruit extract
- Any appropriate buffer
- Colorimeter
- 250 ml beaker
- Muslin cloth
- Water bath maintained at 30°C
- Distilled water

Methods

1. Separate the white of an egg into a 250 ml beaker and add 150 ml distilled water, stirring the mixture thoroughly. The mixture becomes quite cloudy as a result of the denaturation of some of the egg albumen by the water.
2. Heat the mixture until it boils, stirring it regularly.
3. Allow the mixture to cool, and then decant

it through two or three layers of muslin into another beaker. This creates a homogenous milky colloidal solution.

4. Add 2 ml of an appropriate buffer (e.g. pH 7), followed by 2 ml of the albumen substrate solution and 1 ml of fruit extract in a colorimeter tube.
5. Mix the contents of the colorimeter tube, and place it in the colorimeter (previously zeroed using a tube with 4 ml buffer and 1 ml of fruit extract).
6. Read the absorbance and note the time.
7. Place the reaction mixture(s) in a water bath at 30°C to promote the activity of any protease enzymes present.
8. Take further absorbance readings at regular time intervals (say every 5 or 10 minutes), until no further change (decrease) in absorbance is detected.
9. Plot a graph of the change (decline) in absorbance against time. Measuring the time taken for a 50% reduction in absorbance value gives an indication of the protease activity of the original fruit extract.

Observation

The observation for the change in absorbance against time is recorded and plotted on a graph paper.

Results

The protease activity of the fruit was found to be

Precautions

- Fruit pulp should not be over ripe (soft) or too hard.
- Adjust the colorimeter reading to zero using 4 ml of buffer and fruit extract at the beginning.
- Absorbance reading is taken at an equal interval of 5 or 10 min, till it becomes constant or does not change (decrease) or there is 50% reduction in absorbance value.

Viva Questions

- Why is reaction mixture temperature is raised to 30°C?
- Why does a colorimeter absorbance drops with time?
- What is the trend of graph plotted for change in absorbance against time?
- What conclusion can you draw from the trend of the graph?

**Experiment 9****Aim**

Extraction of ethyl alcohol from molasses by yeast fermentation.

Key Terminology

- **Fermentation** A process carried out by yeast (or microorganisms) whereby organic materials are decomposed, with the formation of some chemicals.
- **Yeast** It is a single-celled eukaryotic living organism.
- **Catalyst** A substance that increases the rate of chemical reaction without itself undergoing any permanent chemical change.
- **Decomposition** Breaking down of organic matter into component elements.
- **Froth** A mass of small bubbles in liquid caused by fermentation.
- **WASH** The fermented liquid (containing 15–18% ethyl alcohol).
- **Enzyme fractional distillation** An enzyme, invertase, which is present is added and this acts as a catalyst to convert sugar to WASH. The ethanol is then obtained by fractional distillation.

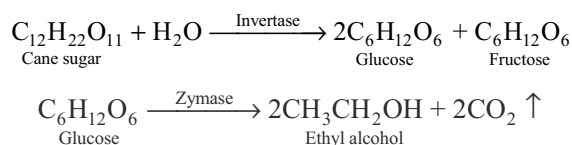
Basic Principle

A solution of glucose in water remains unchanged indefinitely. If, however, solution **yeast** is added to this, after a gap of time the whole liquid starts **frothing** and looks like boiling. On testing it is found that glucose has been converted to ethyl alcohol and the boiling appearance is due to the brisk evolution of carbon dioxide (CO₂) during the reaction.



This process of conversion of sugar to ethyl under the influence of yeast was known since the remotest times and was named **fermentation** (*Ferver*—to boil). It is believed that yeast contains some ‘non-living’ complex organic compounds responsible for this change. These complex compounds bring about fermentation by playing the role of a catalyst. Numerous other decomposition reactions are similarly brought about by complex compounds supplied by yeast or other living organisms. The term fermentation in its modern form covers all such reactions which involve slow decomposition of big molecules of certain organic compounds into simpler forms under catalytic influence of non-living complex substances called “Ferments”. Since number of ferments are present are in yeast, they are known as “enzymes”.

The resulting solution is received in a large fermentation tank (or flask) and yeast is added to it. The mixture is maintained at about 30°C for 2 or 3 days. During this period the enzymes invertase and zymase present in yeast bring about the conversion of sugars into ethyl alcohol. The fermented liquid is known as **WASH**.



To distinguish it from other fermentation reaction, the conversion of sugar into ethyl alcohol by yeast is called **alcoholic fermentation**. In alcoholic fermentation, cane sugar or glucose is the fermenting materials. Therefore, any natural product which contains these sugars or can be easily converted into them is a potential source of

ethyl alcohol. Following are the main two sources for the alcohol industry:

- (i) Substances containing fermentable sugar, e.g. cane sugar, beets, molasses and fruit juice.
- (ii) Substances containing starch, e.g. potatoes, rice, barley and maize. In India most of the commercial alcohol or rectified spirit is prepared from molasses. It contains about 60% fermentable sugars, viz. sucrose, glucose and fructose. In this experiment we will extract ethyl alcohol by alcoholic fermentation of yeast from molasses.

Materials

- Molasses
- Pure yeast culture
- Measuring cylinder
- Test-tube
- Lime water
- Ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$
- Sulphuric acid (H_2SO_4)
- Fractional distillation apparatus
- Conical flask
- Distilled water

Methods

The following steps are involved in extraction of ethyl alcohol by yeast fermentation:

1. **Dilution** Molasses is first diluted with water in 1:5 ratio (i.e. one volume of molasses is mixed with five volumes of water).
2. **Addition of ammonium sulphate** Add ammonium sulphate or ammonium phosphate if necessary (See *Precautions*).
3. **Addition of sulphuric acid** The solution (from Step 1) is then made acidic with a small amount of sulphuric acid. Acidity is favourable to the growth of yeast but unfavourable to most other bacteria.
4. **Addition of yeast** The resulting solution (after adding Ammonium sulphate and H_2SO_4) should be taken in a large fermentation flask and inoculated with pure culture of yeast. The mixture should be incubated at about 30°C for 2 or 3 days.

5. **Fractional distillation** After 3 days the solution should be subjected to fractional distillation in a special column. The column should be divided into a number of compartments by means of plates. These plates are provided with valves for collecting the distillate (Fig. 5.9.1).

The fractional distillation of the WASH yields three fractions:

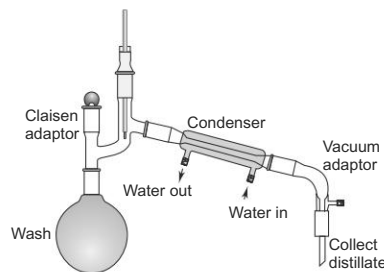


Fig. 5.9.1 Fractional Distillation apparatus

#**Fraction 1:** Low boiling fraction drawn from the head of the column. It consists of **acetaldehyde**.

#**Fraction 2:** Main fraction drawn near the tip of the column. It consists of 95% **ethyl alcohol**

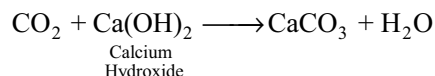
#**Fraction 3:** High boiling fraction ($125\text{--}140^\circ\text{C}$) drawn near the base of the column. It consists mainly of **amyl alcohol** ($\text{C}_5\text{H}_{11}\text{OH}$).

NOTE: Ethyl alcohol can also be prepared from starch, using potato mash and barley malt.

Observations

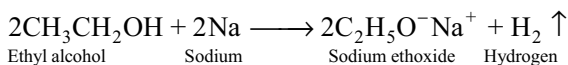
Following two tests will confirm that fermentation has taken place:

(i) **Test for CO_2** After frothing starts (which is an indication for CO_2 liberation, pass the CO_2 gas through lime water and check if it turns milky.



(ii) **Test for Presence of Alcohol** Take about 1 ml of distilled alcohol in a dry test-tube, and add 1 g anhydrous calcium sulphate (CaSO_4) and shake well to remove water. Decant the liquid to another clean, dry test-tube and add a small piece of sodium metal.

A brisk effervescence due to the evolution of hydrogen gas indicates the presence of alcohol.

**Precautions**

- Special care should be taken as ethyl alcohol is highly flammable.
- If the nitrogen content of the molasses is poor, it may be fortified with ammonium sulphate or ammonium phosphate.

Viva Questions

- How do you know fermentation is taking place?
- Which gas does limewater test for?
- Suggest other methods for measuring the speed of this reaction.
- How do you know the presence of ethyl alcohol in the fermented product?

**Experiment 10**

Aim Sauerkraut production and to analyze certain parameters during production (the period of fermentation).

Key Terminology

- **Sauerkraut** Is made by a process of pickling called lactic-fermentation that is analogous to how traditional pickle is made from shredded cabbage.
- **Pectolytic (or protolytic) enzyme** Enzymes that bring about a gradual ripening of fruit causing it to become softer.
- **Anaerobic** Living or active in the absence of free oxygen, e.g. anaerobic bacteria.
- **Hetero-fermentor** It produces CO₂ which replaces entrapped air and helps establish anaerobic conditions within the product and prevents oxidation.

Basic Principle

Sauerkraut means acid cabbage. According to definition it is a clean, sound product of characteristic flavour, obtained by full fermentation, chiefly lactic, of properly prepared and shredded cabbage in the presence of salt. But here, the level of salting is critical for obtaining a satisfactory product; it must be within the range of 2–3% w/w and is normally about 2–25%. Too little salt results

- Care should be taken to avoid excess of acid as the yeast may be killed.
- Selection of pure culture of yeast should be made for inoculation. Avoid infection of resulting solution.
- After inoculation with yeast, check for frothing and conduct test for CO₂ evolution.

in poor flavour and soft kraut, whereas too much salt inhibits the lactic acid bacteria and may result in an acid flavour, darkening and growth of yeast (Pederson, 1979).

Salt serves a number of purposes:

- (i) It extracts moisture from the shredded cabbage by **osmosis to form the brine** in which fermentation will take place. Brine is nothing but a mixture of sugar and nutrients from the cabbage.
- (ii) It helps to inhibit some of the natural microflora of the cabbage such as *Pseudomonas* which would otherwise cause spoilage and helps to select for the lactic acid bacteria.
- (iii) It helps maintain the crisp texture of the cabbage by withdrawing water and **inhibiting endogenous pectolytic enzymes** which cause the product to soften and it finally also contributes to the flavour of the product.

When the glass container is essentially filled with the mixture of salt and cabbage, a plastic sheet is used to cover to keep out dirt and air. This is done to inhibit the growth of yeast and moulds that can result in darkening, softening and development of undesirable flavour.

The process of Sauerkraut fermentation is strongly selective. Because raw cabbage contains substances that are inhibitory to gram-negative bacteria and hence, the presence of inhibitors, an anaerobic environment and salt is responsible for the dominance of lactic acid bacteria population. Underlying this overall dominance by lactic acid bacteria is a natural succession of different species which contribute to the characteristic flavour of sauerkraut. The fermentation is initiated by *Leuconostoc mesenteroids*. As a hetero-fermentor it produces CO₂, acetic acid, lactic acid, alcohol and other products which contribute to the flavour of sauerkraut. The CO₂ produced replaces the entrapped air and helps establish anaerobic conditions within the product and prevents of vitamin C and loss of colour. As the acids accumulate, *L. mesenteroids* is inhibited but the fermentation confirms with *L. Brevis*, *Pediococcus cerevisiae* and *Lactobacillus plantarum*.

In this experiment, fermentation is carried out for days, during which few of the parameters measured are: **odour, colour, pH and total acidity** expressed as percentage of lactic acid.

Materials

- Sample: Head of a cabbage.
- Reagents: (i) 1% of phenolphthalein; (ii) 0.1 N NaOH; (iii) NaCl
- Glassware's: (i) Airtight glass bottle or container; (ii) Burette; (iii) Pipette; (iv) Conical flask

- Equipment: (i) Balance; (ii) pH meter; (iii) Laminar flow; (iv) Autoclave

Methods

1. Remove the outer leaves from the cabbage head and wash with water. The head should be halved and its core removed.
2. Shred the cabbage into pieces of 2–3 mm; shredding the leaves gives a larger total surface area and allows the extraction of juice.
3. Weigh NaCl in amount equal to 3% of the weight of the cabbage and added uniformly to the layers of shredded cabbage. This cabbage and salt mixture should be then placed compactly into a sterile airtight glass container.
4. Keep the container at room temperature and at different intervals, during the fermentation period; examine the cabbage for aroma and colour.
5. A titration of the brine to determine the percentage of lactic acid is done as follows:
 - (a) Remove 2 ml of the fermenting juice and dilute to 10 ml with distilled water.
 - (b) Add 2–3 drops of 1% phenolphthalein to the sample.
 - (c) Titrate the sample with 0.1 N NaOH till a persistent pink colour is obtained.
 - (d) Take mean of 3 readings and calculate the percentage of lactic acid using the formula:

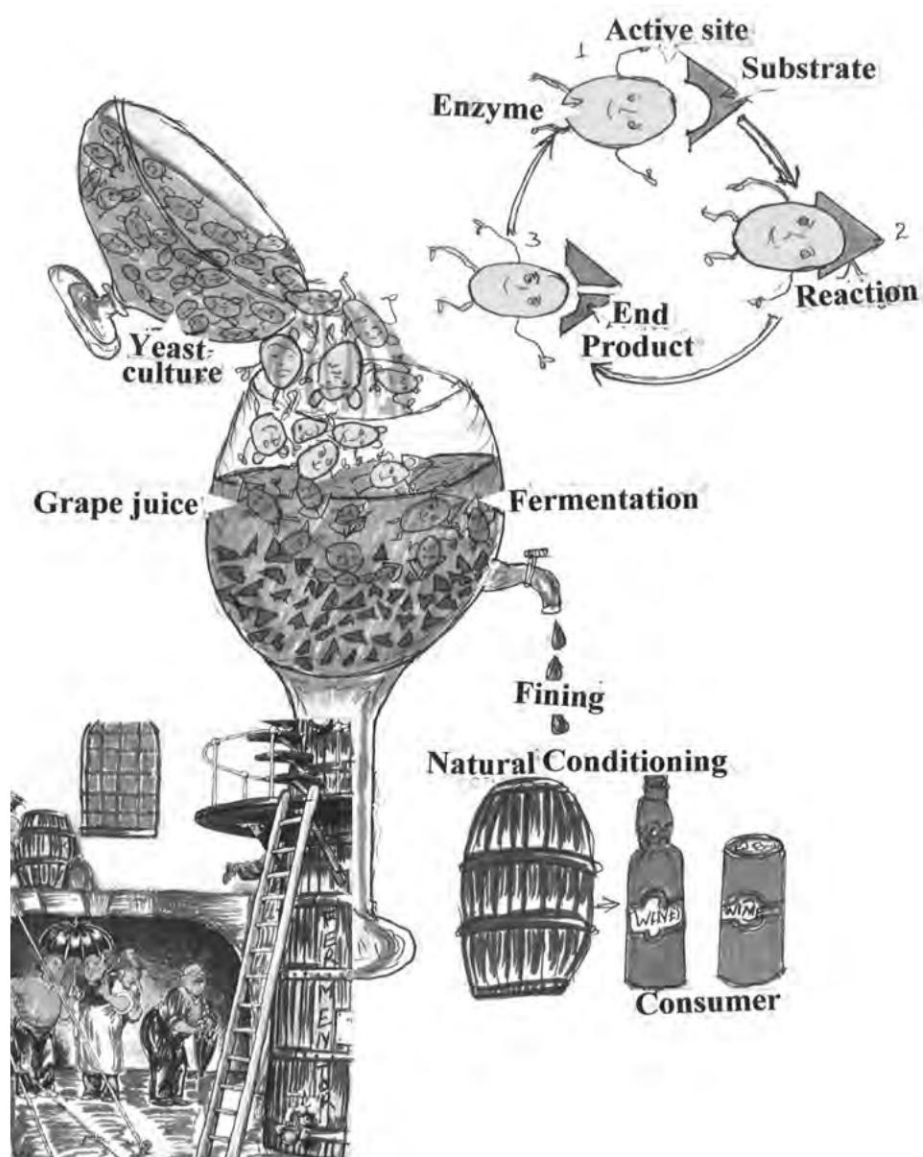
$$\% \text{ lactic acid} = \frac{[(\text{ml of } 0.1 \text{ M NaOH}) \times (0.9)]}{[\text{sample volume}]}$$

Observation

The weight of the cabbage is found to be... gm and the different parameters like colour, aroma, pH and percentage of lactic acid are recorded in the table as follows:

Changes observed during period of fermentation:

Sl. No	Days of fermentation	Parameters			
		Colour*	Odour#	pH	Acidity lactic acid (in %)
1	0				
2	1				
3	2				
4	3				
5	4				
6	5				



6

Cell and Molecular Biology



Experiment I

Aim

Estimation of nucleic acid. To estimate the RNA isolated from a given sample. **OR** Spectrophotometric analysis of RNA/orcinol determination of RNA.

Key Terminology

- **RNA** Ribonucleic acid which contains a ribose sugar moiety.
- **Orcinol** A chromogen which on binding with furfural yields a green colour.
- **Chromogenic substance** A pigment-like substance which when interacts or binds to a specialized molecule gives a detectable colour pigmentation.
- **Spectrophotometer** An instrument with a monochromator which can be used for detection of absorbance or transmission of light through a medium (samples and references kept in a cuvette) at a particular wavelength and the concentration of the samples can be calculated using specific formulae or graphically.

Basic Principle

Detection and quantitative estimation of RNA is based on the principle of the pentose sugar ribose as component of RNA. Ribose or RNA is colourless but if it binds and interacts with specific chromogenic substance, then the intensity of the colour developed can be measured. Ribose, when it interacts with hot concentrated HCl, gets converted to a compound called furfural, which then reacts with a chromogenic substance orcinol to generate a specific green colour. The intensity of this green colour can be measured in a

spectrophotometer at 660 nm from which standard curve can be deduced.

Materials

- RNA
- Alkaline distilled water
- Acid-orcinol reagent
- Boiling water bath
- Spectrophotometer and cuvettes

Chemical Preparation

Acid reagent: To prepare an acid reagent, first dissolve 1g of $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$ in 10 ml of distilled water. From this original concentration take 0.5 ml solution and add to 100 ml of concentrated HCl. Acid reagent should be prepared freshly.

6X alcohol-orcinol (see Annexure II)

Methods

1. Dissolve 0.250 mg of commercial RNA in 250 ml of slightly alkaline distilled water. Use a volumetric flask and proper analytical technique. This will give a standard solution of 1 microgram RNA per ml.
2. Prepare a series of dilutions so that you have 1, 0.5, 0.250, and 0.125 micrograms of RNA per ml.
3. Dissolve your isolated, precipitated RNA in 10.0 ml of alkaline water. Prepare a serial dilution for 1/10, 1/100, 1/1000 and 1/10000.

4. Place 3.0 ml of each standard and 3.0 ml of each serial dilution of the sample RNA into separate test-tubes. Place 3.0 ml of alkaline water in a separate tube.
5. Add 3.0 ml of 6X acid-reagent and 0.3 ml alcohol-orcinol reagent to each tube and mix well.
6. Place the tubes in a boiling water bath for 20 min. Cool the tubes by immersion in an ice bath at the end of the 20 min period.
7. Turn on a spectrophotometer and adjust the wavelength to 660 nm. Blank the spectrophotometer with the alkaline water/orcinol tube. Measure the A_{660} of each of the remaining standards and diluted samples.
8. Plot the absorbance of the standards against the known concentrations. Calculate the concentration of RNA in your sample plotting against the observed absorbance.

Observation

The optical density obtained should be recorded in the table below:

Sl. No.	RNA stock solution (ml)	Corresponding RNA conc. ($\mu\text{g/ml}$)	Absorbance
1			
2			
3			
4			
Unknown 1			
Unknown 2			

Result

The concentration of RNA isolated from _____ was found to be _____ $\mu\text{g/ml}$.

Precautions

- Use fresh acid reagent
- Alkaline water/orcinol should be used as a blank spectrophotometer reading.

Viva Questions

- Why orcinol is used in RNA estimation experiments?
- RNA or ribose sugars are colourless. How does it develop colour to measure in a spectrophotometer?
- How compound furfural develops?
- At which wavelength is the green colour measured in a spectrophotometer.
- Where in the eukaryotic cell two types of nucleic acids are located?
- What are the three major components of a nucleotide and how are they connected?
- What bases are found in nucleic acids? How are the bases classified?
- What kind of carbohydrates are found in nucleotides?
- How do nucleosides differ from nucleotides?
- How do the shapes of DNA and RNA molecules differ?
- What role do DNA and RNA molecules play?
- What kind of bonds form between the bases of DNA or RNA molecules?



Experiment 2

Aim Estimation of DNA by the diphenylamine (DPA) method. **OR** Preparation of DNA standard curve through spectrophotometer analysis using diphenylamine reagent.

Key Terminology

- **DNA** See Experiment 4 in this chapter
- **Spectrophotometer** See Chapter III; Experiment 5
- **Optical Density (OD)** See Chapter III, Experiment 4

Basic Principle

When DNA is treated with **diphenylamine (DPA)** under acidic condition a blue coloured compound is formed with a sharp absorption maximum at 595 nm. This reaction is due to 2-deoxyribose in general and is non-specific for DNA. In acidic solution, the straight chain form of a **deoxypentose is converted to the highly reactive β -hydroxylevulinaldehyde that reacts with diphenylamine to give a blue coloured complex.** In DNA only the deoxyribose or purine nucleotides reacts, so that the value obtained represents half of the total deoxyribose present. The intensity of the blue colour measured at 595 nm is directly proportional to the concentration of sugars cleaved from DNA strands during chemical treatment with DPA.

Materials

- DNA (Herring sperm, commercially available)
- NaCl (0.15M)
- Na-citrate (0.015M)
- Diphenylamine
- Glacial acetic acid
- Sulphuric acid (concentrated)
- Distilled water
- Measuring cylinder

- Chemical storage bottles
- Pipettes
- Test tubes
- Single-beam spectrophotometer

Methods

1. Standard formulation
 - Citrate buffer: (see Annexure II, No. 10)
 - DNA stock solution: Dissolve 10 mg of commercially available herring sperm DNA in 50 ml of citrate buffer and prepare as the stock solution of DNA. Since the prepared solution is not easily soluble, so it can be placed in the water bath maintained at 90°C for 10 min.
 - Diphenylamine reagent (300 ml): Dissolve 3 g of pure DPA in 300 ml of glacial acetic acid and add 7.5 ml of concentrated sulphuric acid to it. This solution should be freshly prepared.
2. Take 5 test-tubes and prepare different DNA stock solution of 0.5 ml, 1.0 ml, 1.5 ml, and 2 ml (in an increasing concentration). Also keep a blank (without DNA).
3. To each test-tube add 4 ml of already prepared DPA reagent mix well and make the volume up to 6 ml. Incubate the tubes in a water bath at 85°C for about 10 min.
4. Transfer the tubes to ice for cooling.
5. Observe the absorbance (OD) of each standard solution in a Spectrophotometer at 595 nm wavelength.
6. Prepare your unknown DNA samples mixed with DPA and follow the steps 3 to 5.

Observation

The OD thus obtained is recorded and tabulated below:

Test-tube	DNA (stock soln. in ml)	Citrate Buffer (ml)	DNA conc. $\mu\text{g/ml}$	DPA (ml)	OD (at 595 nm)
Blank	0	2	0	4	0.000
1	0.5	1.5	100	4	
2	1.0	1.0	200	4	
3	1.5	0.5	300	4	
4	2.0	0.0	400	4	

Note: Based on the table, plot a graph taking OD on the Y-axis and known DNA concentration on X-axis. The graph obtained should be a straight line with the maximum points (at least two) lying on the locus.

Table For Unknown DNA samples

Test tube	OD (at 595 nm)	DNA concentration
Unknown 1		
Unknown 2		
Unknown 3		

Plot the OD observed for the unknown samples against the standard graph prepared earlier and deduce the DNA concentration of the unknown samples.

Result

The concentration of DNA in the Unknown 1, 2, 3 samples were found to be _____ $\mu\text{g/ml}$ respectively.

Precautions

- Use fresh and pure DPA for diphenylamine reagent.
- Thoroughly mix the diphenylamine reagent and incubate in a water bath at 85°C for 10 min or till blue colour develops.

Viva Questions

- How does blue colour complex develop in estimation of DNA by diphenylamine (DPA)?
- Why is DNA treated with DPA under acidic condition?
- Why is developed coloured complex measured at 595 nm in a spectrophotometer?

**Experiment 3****Aim**

Extraction of Genomic DNA. To extract genomic DNA from a given plant specimen by using CTAB method (Based on method of Saghai-Maroo et al., 1984).

Key Terminology

- **Genomic DNA:** Deoxy-ribonucleic acid or DNA is the genetic blueprint of majority of the organisms.
- **Extraction buffer** A balanced solution of chemicals which helps in the homogenization and lysis of the specimen without degrading the genetic material.

- **Ethidium bromide** It is a dye capable of intercalating between the DNA strands and illuminated by UV and can predict the presence of nucleic acid on visualization.

Basic Principle

DNA extraction from plant tissue can vary depending on the material used. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated. Once the tissue has been sufficiently ground, it can then be re-suspended in a suitable buffer, such as CTAB. In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other material are separated through mixing with chloroform and centrifugation. DNA must then be precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA is then re-suspended and stored in TE buffer or sterile distilled water. This method has been shown to give intact genomic DNA from plant tissue. To check the quality of the extracted DNA, a sample is run on an agarose gel, stained with ethidium bromide, and visualised under UV light.

Students often face problem in isolation of a good quality genomic DNA from tissue of various organisms, particularly the ones which possess secondary metabolites (e.g. alkaloids). The method that rely on Cesium chloride (CsCl) gradients are time consuming and expensive. Among the less expensive protocols that give high yields of DNA, CTAB method is the most common and widely used for various plant groups.

Materials

- Tris
- NaCl
- Beta-Mercaptoethanol
- CTAB extraction buffer (*see Annexure II, No. 1*)
- EDTA
- RNase A

- Distilled water
- Centrifuge
- Mortar and pestle
- Octanol
- Chloroform
- 15 ml falcon tubes
- Glass hook

Methods

1. Weigh 500 mg of de-veined leaves and grind it using a mortar and pestle by adding liquid nitrogen. Transfer the ground material to a 15 ml polypropylene tube.
2. Add 9.0 ml of warm (65°C) CTAB extraction buffer to the 500 mg ground tissue. It is best to distribute tissue along the sides of the tube before adding buffer, so as to avoid clumping of dry tissue at the bottom. Mix several times by gentle inversion.
3. Incubate for 60-90 min, with continuous gentle rocking in a 65°C oven.
4. Remove tubes from oven, wait 4–5 min for the tubes to cool down, and then add 4.5 ml chloroform : octanol (24:1). Rock gently to mix for 5–10 min.
5. Spin in a table-top centrifuge for 10 min at 10000 rpm at RT.
6. Pour off top aqueous layer into new 15 ml tubes. Add 4.5 ml chloroform/octanol and rock gently for 5–10 min.
7. Spin in a table-top centrifuge for 10 min at 10000 rpm at RT.
8. Pipette top aqueous layer into new 15 ml tubes containing 30 µl of 10 mg/ml RNase A. Mix by gentle inversion and incubate for 30 min at 37 °C.
9. Add 6.0 ml of isopropanol (2-propanol). Mix by very gentle inversion.
10. Remove precipitated DNA with glass hook.

Observation and Results

The plant genomic DNA is observed as cotton-like threads when precipitated with isopropanol.

Precautions

- CTAB, NaCl, EDTA and Tris HCl should be autoclaved and used.

- The centrifuge tubes used should also be autoclaved and used.
- After precipitation of DNA, it must be washed thoroughly to remove contaminating salts.

Viva Questions

- What is the advantage of CTAB method for isolation of genomic DNA in plants over other methods?
- What is the purpose of the isolation of genomic DNA?
- Why is freshly isolated DNA treated with RNase?

**Experiment 4**

Aim DNA extraction from blood.

Key Terminology

- **White blood cells (WBC)** The nucleated cells of blood containing the nucleic acid material.

Proteinase K A protein degrading enzyme usually used to digest the cell surface of proteins aiding in the lysis of the cells.

DNA Deoxyribonucleic acid or the genetic blueprint of the cell.

Basic Principle

The white blood cells in the whole blood component contains the genomic DNA, whereas the red blood cells are devoid of it. The lysis buffer lyses the red blood cells (RBC's). Proteinase K is then added to digest the proteins. Phenol precipitates out the cell debris and the degraded proteins, leaving out the nucleic acid part in the supernatant, which can be precipitated out with the help of alcohol.

Materials

- Ammonium chloride (NH_4Cl)
- Chloroform
- Ethanol, absolute
- HCl
- Isoamyl alcohol
- Isopropanol phenol
- Potassium carbonate (KHCO_3)
- Proteinase K
- Sodium acetate
- Sodium chloride
- Sodium EDTA (Na_2EDTA)

- Sodium dodecyl sulfate (SDS 10%)
- Tris EDTA (TE) buffer, pH 8.0 (*see Annexure II, No.2*)
- Lysis buffer (*see Annexure II, No. 3*)
- SE-buffer (*see Annexure II, No. 4*)
- Centrifuge
- Micro-pipette
- Eppendorf tubes (1.5 ml)

Chloroform : Isoamyl alcohol 24:1

Chloroform 24 ml

Isoamylalcohol 1 ml

Sodium acetate

3 M Sodium acetate 246 g/l

Adjust to pH 5.2 with CH_3COOH

Proteinase K (10 mg/ml)

Dissolve 100 mg Proteinase K in 10 ml TE buffer for 30 min at room temperature. Aliquot and store at -20°C

Methods

1. Take 200 μl whole blood (collected in EDTA vial) in an eppendorf tube and add 800 μl lysis buffer, shake gently, incubate for 30 min on ice, and centrifuge at 1200 rpm for 10 min at 4°C .
2. Remove the supernatant (blood waste) and add another 800 μl lysis buffer (*see Annexure II, No. 3*), re-suspend the pellet, keep on ice for 10 min, and centrifuge for 10 min at 4°C (1200 rpm).
3. Remove supernatant (blood waste). Add 740 μl SE-buffer (*See Annexure II, No. 4*) and

- re-suspend the pellet, add 10 μl proteinase K (10 mg/ml) and 50 μl 20% SDS shake gently, and incubate overnight at 37°C in a water bath. Alternatively, one may incubate at 56°C for 2 hours.
- Add 750 μl phenol, shake by hand for 10 min, and centrifuge at 10000 rpm for 10 min at RT.
 - Transfer the supernatant into a fresh tube, add 750 μl phenol : chloroform : isoamyl alcohol (25:24:1), shake by hand for 10 min, and centrifuge at 10000 rpm for 10 min at 10°C.
 - Again transfer the supernatant into a new tube, add 750 μl chloroform : isoamyl alcohol (24:1), shake by hand for 10 min, and centrifuge at 10000 rpm for 10 min at 4°C.
 - Transfer the supernatant into a new tube, measure it, and add 1/10th volume 3 M sodium acetate (pH 5.2) and 1 ml isopropanol, shake gently until the DNA precipitated, use a glass pipette, make a hook, and capture the DNA.
 - Wash the DNA in 70% ethanol, centrifuge at 12000 rpm for 10 minutes. Air dry the pellet and dissolve the DNA in 0.1 ml TE-buffer overnight at 4°C on a rotating shaker. (If the DNA is not dissolved leave it longer at 4°C on the rotating shaker.)
 - Measure the DNA concentration in a spectrophotometer and run 2 μl on a 0.8% agarose gel.
- NOTE:** (See Experiment 3; and also consult Chapter I; j Electrophoresis).

Observations

The genomic DNA obtained is initially suspended in an appropriate amount of MQ grade nuclease free water or Tris-EDTA buffer. 3 μl of the re-suspended DNA is reconstituted in 3 ml of nuclease free water, and a blank is also prepared with only water. These preparations are used for spectrophotometric observation at OD = 260 nm and quantitate the concentration of DNA in the extracted resuspended DNA vial. The concentration is calculated using the formula:

$$\text{DNA concentration } (\mu\text{g}/\mu\text{l}) = \text{OD}_{260} \times 50 \times \text{dilution factor}/1000$$

Results

Sample No.	Initial DNA used	Diluent /water used for dilution	Dilution factor	OD ₂₆₀	DNA conc. ($\mu\text{g}/\mu\text{l}$) (OD ₂₆₀ $\times$ 50 $\times$ dilution factor/1000)
SAM. 1 SAM. 2 SAM. 3 SAM. 4 SAM. 5	e.g. 3	e.g. 3000	1000	0.6	30 $\mu\text{g}/\mu\text{l}$

The concentration of the DNA extracted from _____ μl of whole blood sample was found to be _____ $\mu\text{g}/\mu\text{l}$.

Precautions

- Carefully collect blood using a disposable syringe.
- In case the isolated DNA pellet does not dissolve in TE-buffer (See Method, Step 8), leave it for a longer period in the rotating shaker maintained at 4°C.
- Measure the DNA concentration against the standard DNA graph or conversion factor given in Observation.

Viva Questions

- Compare the amount of DNA found in prokaryotes with that found in eukaryotes.
- What special DNA sequences are necessary to propagate, maintain, and correctly distribute pieces of DNA in proliferating eukaryotic cells?
- What happens during DNA replication in prokaryotes, and how does it differ from eukaryotes?
- What conditions are required for initiation of DNA replication in eukaryotes?

**Experiment 5****Aim**

Perform **agarose gel electrophoresis** for screening the DNA isolated and to estimate the relative sizes.

Key Terminology

- **Agarose gel electrophoresis** It is easiest and common method used in biochemistry and molecular biology to separate DNA or RNA molecules by size.
- **Electrophoresis** The migration of charged molecules in an electrical field. A popular way to electrophoretically separate a mixture of proteins is to introduce the sample into a well in a semisolid slab of agarose or starch gel.
- **Ethidium bromide (EtBr)** See *Experiment 3*.
- **DNA ladders** Standard markers representing different sizes of base pair which are co-electrophoresed with DNA samples along with the product to be checked by gel electrophoresis.
- **Restriction enzyme** See *Experiment 4, Chapter VII*.
- **Restriction digestion** See *Experiment 4, Chapter VII*.
- **Size marker** A molecular weight size marker is used to identify the approximate size of a molecule run on a gel electrophoresis. This marker can be composed either of different proteins of known size, used in a **Western Blot**, or can be a nucleic acid that has been digested with a restriction enzyme into fragments of known size, e.g. 100bp marker etc.
- **Gel tracking dye** A colour marker to monitor the process of agarose gel electrophoresis and polyacrylamide gels, e.g. xylene cyanol, bromophenol blue, orange dye, etc.

- **Restriction endonuclease** Any of the enzyme that cleave DNA or cut nucleic acid at specific restriction sites and produce restriction fragments, from bacteria or any other organism.
- **UV-light Box (or Trans-illuminator/gel doc system)** Is the transmission of UV-transmissible light source through tissues of the body. A UV-transilluminator unit such as a box that encloses a UV-light.

Basic Principles

Agarose gel electrophoresis is performed to check the progression of a **restriction digestion**, to quickly determine the yield and purity of a DNA isolation or PCR reaction, and to size fractionate DNA molecules, which then could be eluted from the gel for further molecular biology works.

Agarose gel electrophoresis separates DNA fragments according to their size. As electric current is used to move the DNA molecules across an agarose gel, which is a polysaccharide matrix that functions as a sieve to help catch the molecules as they are transported by the electric current.

Electrophoresis is the movement of charged molecules in an electric field. Negatively-charged molecules like DNA and RNA migrate towards the positive electrode (anode) under the influence of electric current. Electrophoresis through agarose or polyacrylamide gel is the standard method used to separate, identify and purify nucleic acids. They form pores ranging from 100 to 300 nm in diameter, the size depending on the concentration of agarose gel. DNA molecules move through these

pores according to their molecular weight; the rate of electrophoretic movement of DNA molecules increases with decreasing molecular weight.

Before gel casting, dried agarose is weighed out and is dissolved in buffer by heating and the warm gel solution is then poured into a mould (made by wrapping clear tape around and extending above the edges of a glass plate), which is fitted with a well-forming comb. The percentage of agarose in the gel varies depending on the expected size(s) of the fragment(s). EtBr is included in the gel matrix to enable fluorescent visualization of the DNA fragments under UV light. Agarose gels are submerged in electrophoresis buffer in a horizontal electrophoresis apparatus. The DNA/plasmid samples are mixed with gel tracking dye and loaded into the sample wells. Electrophoresis usually is performed at a definite mA current and a set voltage for a specific time period at room temperature, depending on the desired separation. Size markers (DNA ladders) are co-electrophoresed with DNA samples, when appropriate for fragment size determination. For example, lambda phage cleaved with restriction endonuclease HindIII to identify fragments between 2–23 kb, 100 bp marker, etc. After electrophoresis, the gel is placed on a UV light box (trans-illuminator/gel doc system) and a picture of the fluorescent EtBr-stained DNA separation pattern is taken with a polaroid camera.

Table Gel Percentages: Resolution of Linear DNA on Agarose Gels

RECOMMENDED % AGAROSE	OPTIMUM RESOLUTION FOR LINEAR DNA
0.5	1,000–30,000 bp
0.7	800–12,000 bp
1.0	500–10,000 bp
1.2	400–7,000 bp
1.5	200–3,000 bp
2.0	50–2,000 bp

Materials

- TBE (Tris Borate EDTA) (see *Annexure II No. 23 or Chapter 1; page 20*).
- Ethidium bromide solution (10 mg/ml)
- Gel casting tray

- Horizontal gel running apparatus
- Electrophoresis power pack
- Cellophane tape (heat resistant)

Methods

1. Prepare an agarose gel (100 ml), according to the recipes listed below, by combining the agarose and buffer in a 500 ml conical flask, and heating in a microwave for 2–4 minutes until the agarose is dissolved.

	0.8%	1.0%	2.0%
Agarose	0.8 g	1.0 g	2.0 g
10X TBE	10.0 ml	10.0 ml	10.0 ml
ddH ₂ O	90.0 ml	90.0 ml	90.0 ml
EtBr (10 mg/ml)*	10 µl	10 µl	10 µl
Total volume	100 ml	100 ml	100 ml

*Add EtBr after dissolving the agarose; swirl to mix, and pour the gel onto a taped plate with casting combs in place. Allow 20–30 min for solidification. (See page 21 and 24)

2. Carefully remove the tape and the gel casting combs and place the gel in a horizontal electrophoresis apparatus. Add 1X TBE electrophoresis buffer to the reservoirs until the buffer just covers the agarose gel.
3. Add at least one-tenth volume of 10X agarose gel loading dye to each DNA sample, mix, and load into the wells. Perform electrophoresis of the gel at 60–80 V until the required separation has been achieved, usually 20 mins–1 hour. Visualize the DNA fragments on a long wave UV light box and photograph with a Polaroid camera.
4. Dispose the gel in a biohazard bin.

NOTE: (also consult Chapter I, j. Electrophoresis).

Observation

Visualize the orange-colour band in the gel representing the presence of DNA under UV light using a transilluminator or gel documentation system.

Results

The presence of the orange colour band in the agarose gel electrophoresis symbolizes the successful extraction of DNA from the source material.



Experiment 6A

Aim RNA isolation and cDNA preparation. To isolate total RNA from liver tissue by Trizol method.

Key Terminology

- **RNA** Ribonucleic acid which contains a ribose sugar moiety.
- **Optical Density (OD)** See Chapter III, Experiment 4.
- **Reverse transcriptase** It is an RNA-dependent DNA polymerase which catalyzes the reaction of conversion of RNA into a complementary DNA (cDNA) which is much more stable than RNA.

Basic Principle

Trizol reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a monophasic solution of phenol and guanidine isothiocyanate, is an improvement to the single step RNA isolation method developed by Chomizynski and Sacchi. During sample homogenization or lysis, trizol reagent maintains the integrity of RNA while disrupting cell and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. After transfer of the aqueous phase, RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and the protein in the sample can be recovered by sequential precipitation.

Materials

- Trizol reagent
- Chloroform
- Isopropanol
- 70% ethanol
- DEPC (Diethyl pyrocarbonate) treated water
- Centrifuge
- Vortex
- Eppendorf tubes
- Micropipettes
- Mortar and pastel

Methods

1. Take a small piece of liver (chicken/mutton), etc. and crush with the help of a mortar and pestle by adding liquid nitrogen. This will result in powdered liver extract.
2. Add 1 ml of trizol to the crushed powdered liver tissue, mix, transfer the aqueous powdered extract to an eppendorf tube. Keep it at room temperature for 10 min.
3. Add 200 μ l chloroform, vortex briefly and keep at room temperature for 10 min.
4. Centrifuge at 13000 rpm for 15 min at 4°C. Transfer the aqueous upper layer to a fresh eppendorf tube, and add 600 μ l of ice cold isopropanol.
5. Keep the tube for incubation at -20°C for 20 min.
6. Centrifuge at 13000 rpm for 10 min at 4°C. RNA precipitates as a pellet. Decant and throw the supernatant slowly retaining the pellet.
7. Add 500 μ l of 70% ethanol and suspend the pellet by tapping slowly.
8. Centrifuge at 13000 rpm for 10 min at 4°C. Decant and discard the supernatant. Air dry the pellet, and finally re-suspend in 30 μ l of Tris-EDTA buffer or nuclease free water.
9. Quantify the RNA by spectrophotometer at 260 nm and adequate dilution.

Observation

$$\text{RNA conc. } (\mu\text{g}/\mu\text{l}) = \frac{\text{OD}_{260} \times 40 \times \text{dilution factor}}{1000}$$

The quantified RNA is then used for cDNA preparation (Experiment 6B).

Results

The concentration of the total RNA isolated from the liver of _____ was found to be _____ $\mu\text{g}/\mu\text{l}$.

Precautions

- Total RNA is highly unstable and can be degraded by any forms of RNAase, dust, etc. Therefore, one has to be extremely careful while handling RNA and the place of work should be absolutely clean while doing the isolation. One must use fresh gloves while isolating RNA.
- All the tips and tubes used for isolation should be double autoclaved.
- Any buffers to be prepared for RNA isolation should be made using DEPC treated autoclaved water only.

Viva Questions

- Why is a trizol reagent used in preparation of cDNA?
- Why is crude extract of the tissue purified?
- What are the contaminants that interfere in RNA extraction?
- What is a monophasic solution? What are the chemicals it consists of?

**Experiment 6B****Aim**

Complimentary DNA (cDNA) preparation from the isolated total RNA.

Key Terminology

- **DNA polymerase** An enzyme that catalyzes the phosphodiester bonds in the formation of DNA, also known as reverse transcriptase.

Basic Principle

RNA is highly unstable in nature and can be potentially degraded by a number of external agents and factors. In order to save the RNA, it needs to be converted to a much stable form, i.e. cDNA. This is achieved through the action of RNA dependent DNA polymerase called reverse transcriptase, which uses the mRNA component of

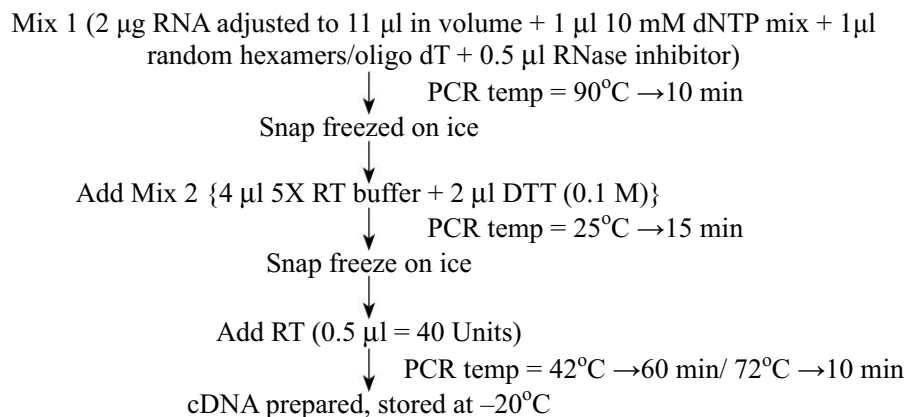
the total RNA to convert it to a much stable cDNA for further molecular biology work.

Materials

- Isolated quantified total RNA
- 10 mM dNTP
- RNase inhibitor
- Random hexamer
- Reverse transcriptase
- 5X RT buffer (*See Annexure II, No. 19*)
- 0.1 M DTT
- PCR
- Ice

Methods

1. Prepare the mix under chilled condition. 2 µg of the total RNA may be used for cDNA preparation (20 µl total) using PCR which is represented through a flow chart below:



Result

The cDNA was prepared using the total RNA isolated from _____ as template, and stored at -20°C for further molecular biology analysis.

Precautions

- Purification of crude RNA extract from a tissue or cell type is very essential to avoid interference.

Viva Questions

- From where is the cDNA prepared?
- In an electrical field, in which pole will DNA move?
- Which is more unstable RNA or cDNA?
- What is the use of PCR in cDNA preparation?
- How is the second strand of cDNA synthesized?
- What are the two methods used in cDNA synthesis of the second strand?
- What is the role of reverse transcriptase in preparation of cDNA?
- What is a cDNA Library?

**Experiment 7A****Aim**

Fractionation of cellular components—Isolation of mitochondria.

Key Terminology

- **Mitochondria** The ATP generating power-house organelle of the cell.
- **Centrifugation** It is a process used to separate or concentrate materials suspended in a liquid medium. The theoretical basis of this technique is the effect of gravity on particles (including macromolecules) in suspension. Two particles of different masses will settle in a tube at different rates in response to gravity.
- **Homogenization** Mechanical and chemical disruption of cells or tissues resulting in the lysis of the cells and extraction of the cellular organelles, nucleic acids and proteins, etc.
- **Cellular organelles** Organelles that allow the cell to function properly. The study of biogenesis of these organelles.

Basic Principle

The basic steps involved in the isolation of mitochondria are (i) rupturing of cells by mechanical and/or chemical means, (ii) differential centrifugation at low speed to remove debris and extremely large cellular organelles, and (iii) centrifugation at a higher speed to isolate and collect mitochondria.

Materials

- A suitable source of fresh liver, e.g. chicken, goat, or rat liver
- 0.25 M sucrose in 10 mM HEPES buffer, pH 7.5 (or homogenization buffer; *See Annexure II, No. 11*).
- 0.25 M sucrose in 10 mM HEPES, pH 7.5 + 1 mM EDTA (or Suspension buffer; *See Annexure II, No. 7*)
- Teflon homogenizer
- Refrigerated centrifuge
- Janus Green B solution
- Hemocytometer and microscope

Methods

1. Take a piece of fresh liver and weigh it.
2. Place the liver in a beaker, and for each gram of liver, add 9.0 ml of 0.25 M sucrose in 10 mM HEPES buffer (*See Annexure II, No. 11*), pH 7.5 and crush. This will produce a 10% homogenized suspension.
3. Shift the homogenized suspension to centrifuge tubes and centrifuge at 5000 rpm for 10 min at 4°C .
4. Decant the supernatant into clean centrifuge tubes and discard the pellet.

5. Recentrifuge the supernatant at 14,000 rpm for 25 min at 4°C.
6. Decant and discard the supernatant. Resuspend the pelleted mitochondria in 20 ml of 0.25 M sucrose in 10 mM HEPES (*See Annexure II, No. 11*), + 1 mM EDTA.
7. Recentrifuge the suspended pellet at 14,000 rpm for 25 minutes at 4°C.
8. Decant and discard the supernatant, and resuspend the washed pellet in 20 ml of fresh sucrose (without EDTA) and place the suspension in an ice bath.
9. Mix a few drops of Janus Green B solution with 0.1 ml of mitochondrial suspension. Place one drop of this mixture in a hemocytometer and determine the number of mitochondria per ml. If there are too many mitochondria to count, make serial dilutions of 1/10 to 1/1000 and recount.

Observations

Take average of large (Minimum 20) number of observation.

Viva Questions

- What are the functions of mitochondria in eukaryotic cells?
- Why is hemocytometer used?
- What are the functions of HEPES buffer and EDTA buffer in mitochondria isolation?

Record the number of mitochondria/ml

Result

The number of mitochondria per ml of the mitochondrial suspension isolated from _____ liver was found to be _____

Precautions

- The liver piece used should be as fresh as possible.
- It is advisable to use a rat liver which has not been fed for the last 24 hours before sacrifice, under sanitized laboratory conditions.
- Steps 7 and 8 can be omitted if a cleaner mitochondrial preparation is not required. If so then use only 0.25 M sucrose in 10 mM HEPES solution for re-suspension.
- Diluted mitochondria must be counted rapidly. They are not stable and will decompose if not counted within a few minutes of the dilution.

**Experiment 7B****Aim**

Isolation of chloroplasts from a given plant specimen.

Key Terminology

- **Chloroplast** Site of photosynthesis for plants.

Basic Principles

Isolation of chloroplasts is done through mechanical cell wall and cell membrane breakage, removal of cell debris and unbroken leaf tissue by filtration and collecting total cell chloroplasts by density gradient ultracentrifugation.

Materials

- Fresh hibiscus or spinach leaves
- Grinding solution (*see Annexure II, No. 6*)

- Chopping board and knife
- Chilled mortar and pestle
- Cheesecloth
- Refrigerated preparative centrifuge
- Suspension solution (*see Annexure II, No. 7*)
- Hemocytometer and microscope

Methods

1. Pre-cool all glassware to be used.
2. Take few fresh leaves and remove any large veins by tearing them loose from the leaves. Weigh out 5.0g of de-veined leaves; and chop them finely.

3. Add the chopped leaves to a pre-chilled mortar containing 20 ml of grinding solution and grind to a fine paste.
4. Filter the solution through a double layered cheesecloth into a beaker and squeeze the leaves pulp to recover all the filtrate.
5. Transfer the filtrate to a cold 50 ml centrifuge tube and centrifuge at 2000 rpm for 1 min at 4°C to pellet the unbroken cells and fragments.
6. Transfer the supernatant into a clean centrifuge tube and re-centrifuge at 10000 rpm for 10 min. The pellet formed during this centrifugation contains chloroplasts. Decant and discard the supernatant.
7. Resuspend the chloroplast pellet in 3–5.0 ml of cold suspension solution or 0.035 M

NaCl. Gently disrupt the packed pellet. This is the chloroplast suspension. Cover the chloroplast suspension with an aluminium foil and place it on ice.

8. Determine the number of chloroplasts/ml of suspension media using a hemocytometer.

Observation

Take average of large (Minimum 20) number of observation.

Result

Record the number of chloroplast per ml of the chloroplast suspension isolated from _____
Plant leaves was found to be _____

Viva Questions

- What are the functions of chloroplasts in plants?
- What are the different types of chloroplasts found in plants?



Experiment 8

Aim

Separation of proteins by SDS gel electrophoresis.

Key Terminology

- **SDS** Sodium Dodecyl Sulfate (also called lauryl sulfate) is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide pH range.
- **Electrophoresis** See *Experiment 5, Chapter 6*.
- **Polyacrylamide gel**: A polyacrylamide gel is a separation matrix used in electrophoresis of biomolecules, such as proteins or DNA fragments.
- **Polymerization**: It is a process of reacting monomer molecules together in a chemical reaction to form three-dimensional networks or polymer chains.
- **Anionic detergent** These are common dissociating agents used to denature or unfold native proteins to individual polypeptides, e.g. sodium dodecyl sulfate (SDS); and also

gives them a near uniform negative charge along the length of the polypeptide.

- **Isoenzymes** There are enzymes that differ in amino acid sequence but catalyze the same chemical reaction.
- **Hydrophilic** These have a tendency to dissolve in water or when wetted by water.
- **Anionic detergent** A class of synthetic detergents in which the molecules do not ionize in aqueous solutions.
- **Western blotting**: The western blot (also called protein immunoblot) is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions). The proteins are then transferred to a nitrocellu-

lose membrane, where they are probed (detected) using antibodies specific to the target protein.

- **SDS-PAGE:** Electrophoresis of proteins done on a denaturing type of polyacrylamide gel because of the presence of the anionic detergent SDS.

Basic Principle

When separated on a polyacrylamide gel, the procedure is abbreviated as **SDS-PAGE** (for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis). The technique is a standard means for separating proteins according to their molecular weight. Polyacrylamide gels are formed from the polymerization of two compounds, **acrylamide** and **N, N-methylenebisacrylamide (Bis, for short)**. Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of **ammonium persulfate along with either DMAP or TEMED**. The gels are neutral, **hydrophilic**, three-dimensional networks of long hydrocarbons cross-linked by methylene groups. The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The pore size of a gel is determined by two factors: the total **amount of acrylamide present** (designated as %T) and the **amount of cross-linker (%C)**. As the total amount of **acrylamide increases, the pore size decreases**. With **crosslinking, 5%C gives the smallest pore size**. Any increase or decrease in %C increases the pore size. Gels are designated as percent solutions and will have two necessary parameters. The total acrylamide is given as a percentage (w/v) of the acrylamide plus the bis-acrylamide.

SDS (also called sodium **lauryl sulfate**) is an **anionic detergent**, meaning that when dissolved its molecules have a net negative charge within a wide pH range. A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass. The **negative charges on SDS destroys most of the complex structure of proteins**, and are strongly **attracted towards an anode** (positively-charged electrode) in an electric field, i.e., electrophoresis. Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured

polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides. In a gel of uniform density the relative migration distance of a protein (R_f) is negatively proportional to the log of its mass.

Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions. The purity of protein samples can be assessed and the progress of a **fractionation or purification procedure** can be followed. Different staining methods can be used to detect rare proteins and to learn something about their biochemical properties. Specialized techniques such as **Western blotting, two-dimensional gel electrophoresis**, and peptide mapping can be used to detect extremely scarce gene products, to find similarities among them, and to detect and separate **isoenzymes** of proteins.

Materials

30% Acrylamide solution stock

Acrylamide	29.2 g
Bis-acrylamide	0.8 g
ddH ₂ O	100 ml

Keep at 4°C,

Tris.HCl (pH 8.3) 0.5 M + 0.4% SDS (adjust pH before adding SDS). Keep at RT.

Tris.HCl (pH 6.8) 0.5 M + 0.4% SDS (adjust pH before adding SDS). Keep at RT.

Ammonium Persulfate (APS)–10%. Keep at 4°C less than 1 month.

Methods

Also consult Experiment 5 of this chapter and also consult Chapter I; j Electrophoresis and page 21).

A standard working volume of constituents to be used for mid-size SDS-PAGE electrophoresis is tabulated below:

1. Assemble glass plates and sealers using clamps. Be sure the sealers are at the appropriate position between the two glass plates to avoid leaking.
2. Prepare the separating gel mixture depending on the required strength of the gel as per the table below:

Separating gel

CONSTITUENTS	GEL PERCENTAGE			
	14%	12%	10%	8%
30% acrylamide	7 ml	6 ml	5 ml	4 ml
4X Tris (pH 8.8)	3.75 ml	3.75 ml	3.75 ml	3.75 ml
H ₂ O (MQ)	volume adjusted up to 15 ml			
10% APS	125 µl	125 µl	125 µl	125 µl
TEMED	12 µl	12 µl	12 µl	12 µl

Add TEMED and APS at the end. Gently swirl the flask to mix, being careful not to generate bubbles. Pipette the solution to a level of 4 cm of the top. Add 0.3 ml of n-butanol. Let polymerization of the gel to take place for another half an hour at least. Rinse the surface of the gel with water before pouring the stacking gel.

3. Prepare the stacking gel by mixing the components listed in the table below:

Stacking gel

CONSTITUENTS	AMOUNT
30% acrylamide	0.65 ml
8X Tris.Cl/SDS (pH 6.8)	0.625 ml
H ₂ O (MQ)	Make volume up to 5 ml
10% APS	25 µl
TEMED	7 µl

Fill each sandwich with stacking gel solution and insert a comb into each place taking care not to trap any bubbles below the teeth. The gel should fully polymerize after 30–45 min.

4. Measure out 15–18 µg of protein from each sample and add 3 µl of Laemmli buffer 2X (*Annexure II, No. 13*) in a vial (eppendorf tube), and denature the sample by subjecting it to heating in a thermal block/PCR/water bath at 95–100°C.

(Prior to adding the sample buffer, keep samples at 0°C. Add the SDS sample buffer to the sample (still on ice), and boil at 100°C immediately 3 to 5 min).

Load the samples in the SDS-PAGE in the respective wells. Load one of the wells

with 0.5–1 µg of standard protein ladder for comparison.

Allow the SDS-PAGE to run at 60 V for 90 min or till the blue front comes out of the gel in a running buffer (Tris-3 g, Glycine-14.4 g, 20% SDS-5 ml adjusted up to 1 l by adding MQ water). Once the run is complete, take out the gel carefully and perform the staining of the gel.

Gel staining

1. After taking out the gel, put it straight into the Coomassie Brilliant Blue staining buffer. Stain overnight at RT on a platform shaker in dark.

Gel staining solution

Methanol CP	500 ml	50%	Methanol CP
Acetic acid CP	100 ml	10%	Acetic acid CP
H ₂ O	400 ml	40%	H ₂ O
Coomassie Brilliant Blue R	2.5 g	0.25%	Coomassie Brilliant Blue R

2. Carefully decant out the gel staining solution (may be reused) and wash with water 2–3 times and destain by several changes of destaining solution.

Destaining solution

Methanol	150 ml	15%
Acetic acid	100 ml	10%
H ₂ O	750 ml	75%

Observation

One may view the blue-coloured protein bands and compare the size with the protein ladder, or the expression compared to a standard control.

Result

The protein extracted from the _____
Sample analyzed by SDS gel electrophoresis
showed presence of protein bands at _____
kilo Daltons.

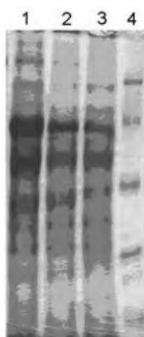


Fig. 6.8.1 A representative SDS gel stained with Coomassie Brilliant Blue. The first three lanes represent protein bands of various sizes of different samples, and the fourth lane is a standard protein ladder for comparison

Precautions

- **All the chemicals used in this experiment are harmful (may cause irritation to the skin). Use gloves and mask.**
- The sealers should be placed at the appropriate positions to avoid leakage.
- Acrylamide is a toxic substance so use with care and wear gloves while handling solutions that contain it. Use in a well ventilated area, and report any spills. Stock solutions should be kept in a fume hood.
- An Erlenmeyer flask is good for mixing acrylamide, since the narrow neck can be stoppered to prevent toxic fumes from escaping.
- After adding TEMED and APS, swirl the flask gently, so that air bubbles are not generated.
- Run the SDS-PAGE at a constant voltage (without any current fluctuation), till the blue bands do not come out of the gel.

Viva Questions

- For what kind of estimation SDS-PAGE protein separation is used?
- How is the pore size of the gel controlled?
- How are polyacrylamide gels formed? Name the compounds.
- What is the separation of protein molecules dependent upon?
- What is an isoenzyme of protein?
- What is the name used for two-dimensional electrophoresis and peptide mapping technique?

**Experiment 9****Aim**

Blood smear preparation and screening. To prepare a blood smear and to study the types of cell present.

Basic Principle

Giemsa's stain is used for staining cells in blood. Giemsa's stain contains both acidic and basic dyes and therefore stains the granules of both eosinophils and basophiles.

Different types of cells found in blood are as follows:

1. **RBC or Erythrocyte:** These appear as a circular biconcave, non-nucleated disc-like structure. They are present in abundance. RBCs contain a homogenous cytoplasm having a diameter of about 0.7 μm . In group

they appear pink in colour.

2. **(a) WBC or Leukocyte:** They have no pigment in their cytoplasm and when unstained are grey coloured. There are two different types of leucocytes on the basis of their cytoplasm.

(b) Granulocyte or polymorphonuclear cells (PMN)

These cells have a granular cytoplasm with many lysosome that contains digestive enzymes. It has a multilobed, uninucleated nucleus.

Depending upon how it stains the granules the cells are classified into three types:

- (i) *Neutrophil*: It stains with neutral dyes cytoplasm of these cells contain fine granules and nucleus in multilobed having a diameter of about $12\ \mu$
- (ii) *Eosinophil*: They have red cytoplasmic granular stain with an acidic dye as eosin. The cell membrane is delicate and the nucleus is generally bilobed (spectacle shaped nucleus)
- (iii) *Basophiles*: The nucleus is slightly trilobed. The cytoplasmic granules are big and blue in colour which is stained with basic dyes.

(c) Agranulocyte

These cells do not possess granules in their cytoplasm. They are uninucleated and without lobes. These are of two types:

- (i) *Lymphocytes*: It is uninucleated which occupy the major part in the cytoplasm. The cytoplasm is homogenous and scanty. Their sizes vary from $10\text{--}14\ \mu$ in diameter.
- (ii) *Monocyte*: They are bigger than lymphocyte and neutrophil. The nucleus is uninucleated and kidney shaped.

Materials

- Blood sample
- Spirit
- Methanol
- Giemsa's stain
- Slides
- Sterilized needle
- Microscope

Methods

1. Take a drop of blood on a clean slide by pricking one's finger.
2. With another slide the smooth edge held at 45° angle to the first slide over the drop of blood, spread over the surface of the first slide to make a thin film of the blood.
3. The thin film should be allowed to air dry.
4. Dip the slide in methanol for two minutes to fix the smear.
5. Add Giemsa's stain (2% in methanol) to the slide and leave for about 10 min.

6. After 10 min, drain of the stain and wash with running tap water, till the smear shows up as a pink film.
7. Observe the slide under microscope at 40x and 100x.

Observations

The blood cells observed under the microscope at 40x and 100x and are classified as different cell types depending upon their morphology and staining pattern.

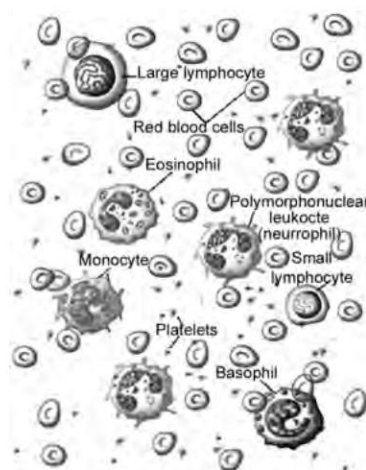


Fig. 6.9.1 Representative photograph showing the morphology of different cell types observed under the microscope

Result

The blood smear showed _____ different kinds of blood cells when observed under microscope, from the prepared blood smear on the slides. These were _____

Precaution

- Care must be taken not to over stain the blood smear.
- Blood film should be made in a single swift action.
- The edge of the spreading slide must be very smooth.
- The angle between the two slides must be approximately 45° .
- Disposable syringe and needle should be used for collection of blood.

Viva Questions

- What are the three types of granulocytes, which can be differentiated depending on the stain?
- How many nuclei do we find in lymphocytes and monocytes?
- How does RBC differ from a WBC?

**Experiment 10**

Aim Problems in molecular genetics.

Nucleic Acid Chemistry

Q1. Draw your conclusion about the nucleic acids in the table given below:

NUCLEIC ACID MOLECULE	%A	%T	%G	%C	%U
a	26	26	24	24	0
b	41	0	41	7	11
c	17	17	33	33	0

Q2. In a double-stranded DNA molecule containing 26% G (guanosine).

- What will be the complete base composition of this particular molecule?
- What will be your answer for the same question, if we assume the molecule is a double-stranded RNA?

Q3. DNA Replication—the enzymology

- What is a primosome and a replisome? What enzymes make up each of them?
- What is the relationship between these structures?

DNA Transcription

Q4. In a double-stranded DNA, following is the sequence of bases representing the start of a gene.

- What will be the sequence of the transcribed RNA, and its polarity?
- What will be the polarity of the DNA?
GCTACGGATTGCTG
CGATGCCTAACGAC

Q5. Find out the sequence of both strands of the DNA, from which the following RNA was transcribed. Determine the 5' and 3' ends of the DNA, and with an arrow sign indicate which strand was transcribed.

5'-CCAUCAUGACAGACCCUUGCUAACGC-3'

Q6. Following is a drawing of a eukaryotic gene. Solid bold lines represent the coding region and broken lines represent introns. If cytoplasmic messenger RNA is hybridized to nuclear DNA, what will be the RNA-DNA hybrid like? Represent your answer by a drawing.

Q7. Enzyme exonuclease III, removes bases from the 3' end of double stranded DNA, sequentially. The following molecules, each 150 bp long, are digested by exonuclease III. Molecule 1 is completely digested, and molecule 2 is only partially digested. Explain the results.

Molecule 1: CGTTCAG.....

GCAAGTC.....

Molecule 2: AAAAAAAAAA.....

TTTTTTTTTTT.....

Restriction Mapping

Q8. A linear DNA molecule 2000 base pair (bp) when digested with the following restriction enzymes, produced the following results:

*eco*RI 800bp, 1200bp
bg/II 500bp, 1500bp
*eco*RI + *bg*/II 500bp, 700bp, 899bp
 Draw the restriction map.

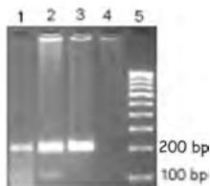
Q9. A 2000 base pair (bp) long linear DNA molecule gives the following size fragments when treated with the following restriction enzymes.

*eco*RI: 600bp, 1400bp
*bam*HI: 1150bp, 850bp

*eco*RI + *bam*HI: 850bp, 550bp, 600bp
Describe the restriction map.

Genetic Code

- Q10. If a one-base mutation (e.g. at A, U or G) occurs in methionine (AUG), what different amino acids could result?
- Q11. Suppose Madan and Mohan are brothers with identical genetic makeup. Madhav is Mohan's friend but has a different genetic makeup in the sense that he has a polymorphism in one of the alleles for a specific gene 'Z'. All the 3 persons' Z gene was checked for heterozygosity by regular PCR-RFLP method, and analyzed by an agarose gel which gave the following gel picture.



Given that the normal PCR-RFLP products yield 200 bp product, and lane 5 is a standard DNA ladder with the lowest band of 100 bp, which lane represents Madhav's case?

- (a) Lane 4 (b) Lane 2

- (c) Lane 3 (d) Lane 1

Proteins

- Q12. Which of the following is NOT a major grouping of proteins in cells?
- enzymes
 - antibodies
 - energy storage units
 - structural units
 - ALL of the above are major classes.
- Q13. Which protocol is used for analyzing the expression of protein?
- Northern blotting
 - Southern blotting
 - Western blotting
- Q14. The formation of the peptide bond is catalyzed by
- EF-Tu
 - aminoacyl tRNA synthetase
 - peptidyl transferase
 - GTP
 - Tryptophan.
- Q15. The charged initiator tRNA binds initially to the ___ in protein synthesis.
- P site
 - UGA base sequence of mRNA
 - E site
 - 3' end of mRNA
 - A site

Note: For restriction digestion mapping refer to experiment No. 4 of Chapter 7, page No 191–192

Notes and Sketches





7

Recombinant DNA Technology



Experiment I

Aim **Bacterial Transformation.** Competent cell preparation. Bacterial transformation and screening of transformed colonies.

Key Terminology

- **Transformation** A mechanism of genetic transfer between bacterial cell in which the donor DNA is cell-free DNA in the recipient bacterium's immediate environment.
- **Competent cell** The ability of a recipient bacterium to take up free DNA and become transformed. Competence is an inheritable characteristic of certain strains of bacteria.
- ***Escherichia coli*** A gram negative bacteria which are grouped as coliforms, but are also utilized as a useful tool in genetic engineering and molecular biology based research works as they can be easily genetically manipulated.
- **Plasmid** Extra-chromosomal DNA present in bacteria coding for antibiotic resistance etc., which may also be used as a designed vector system for gene delivery.
- **DH5α strain** A genetically modified *Escherichia coli* strain.

Basic Principles

Escherichia coli cells are grown to log phase (See Chapter III; Experiment 3). Cells are concentrated by centrifugation and re-suspended in a solution containing calcium chloride. For efficient uptake, the bacteria have to undergo some form of physical and/or chemical treatment that enhances their ability to take up external DNA. Exposure to calcium ions renders the cell's ability to take up DNA, or 'competent'. Plasmid DNA is mixed

with the cells and presumably adheres to them. The mixture of DNA and cells is then heat shocked, which allows the DNA to efficiently enter the cells. The cells are grown in nonselective medium to allow synthesis of plasmid-encoded antibiotic resistance proteins, and then plated on antibiotic-containing medium to allow identification of plasmid-containing colonies.

The fact that *E. coli* cells that are soaked in an ice-cold solution are more efficient at DNA uptake than unsoaked cells is used to make competent *E. coli*. Traditionally, a solution of CaCl_2 is used for this purpose.

Materials

- DH5α strain
- LB broth (See Annexure III, No. 10 & 11)
- Ochrich tube/Falcon tubes
- Calcium chloride
- Centrifuge
- Magnesium chloride
- Magnesium sulphate
- Suitable antibiotics, e.g. ampicillin (100 mg/ml)

Methods

Preparation of Competent Cells

1. A genetically modified strain of *E. coli* (e.g. **DH5α** etc.) should be streaked on LB agar plate and grown overnight at 37°C.

2. Next day 7–8 single colonies from the plate should be inoculated into 1 ml LB separately and allow to grow by incubating the cultures at 37°C in a water bath or incubator. Always keep a negative control.
3. Transfer the inoculums showing growth to 50 ml LB broth containing 20 mM MgSO₄ and MgCl₂ and grown as shaking cultures at 37°C until the absorbance at 590 nm becomes 0.3–0.4.
4. Harvest the cells by transferring the culture in Ochrich/plastic falcon tubes and subjecting to centrifugation at 4000 rpm for 15 mins. Decant the supernatant carefully and retain the precipitated bacterial cells in the tube.
5. Chill the cells by placing them on ice for 30 minutes.
6. Resuspend the pellet in 1/3 volume of ice-cold 0.1 M CaCl₂. Keep on ice for an additional 30 minutes and then centrifuge at 4000 rpm at 4°C for 15 minutes.
7. Discard the supernatant and re-suspended pellet into 2 ml of ice-cold 100 mM CaCl₂. Competent cells can be stored in 100 mM CaCl₂ + 15% sterile glycerol (optional) at –70°C in 200 µl aliquots.
4. Allow the cells to chill on ice for 5 minutes.
5. Add 1 ml of LB broth (*See Annexure III Nos.10 & 11*) and incubate at 37°C for 45 minutes with shaking to allow the bacteria to recover and express the antibiotic (e.g. ampicillin) resistance marker encoded by the plasmid.

Observations

1. Take 150 µl and 50 µl aliquots of the transformation mixture and plate on LB plates (with added suitable antibiotics) and gently spread on the entire plate by slight tilt.
2. The plate should be incubated at 37°C for 16 h. Remember to keep a negative control plate with added antibiotic.
3. Next day, single colonies are observed if the transformation is successful. One may pick up few colonies separately and grown in LB broth, from which he/she may either go for plasmid isolation (*See Experiment 2*), or prepare a glycerol stock of the bacterial cell containing the plasmid and store at –70°C or liquid nitrogen.

Results

Colonies when grown in LB-broth (with added antibiotics), shows bacterial growth, indicating transformation has taken place.

Precautions

- Always maintain a negative control, i.e. a culture without inoculation, to check for any unwanted contamination while handling.
- Keep proper labels to differentiate between control and antibiotic resistant transformed cultures.

Transformation of Plasmid and Screening of Transformed Colonies

1. Thaw the competent cells previously prepared on ice.
2. Mix 200 µl of competent cells with plasmid provided (50 ng in a volume of 10 µl) in a clean chilled eppendorf tube on ice and store on ice for 30 minutes.
3. Give a heat shock at 42°C for 90–120 seconds in a water bath (pre-set) and tubes should be rapidly transferred back to ice.

Viva Questions

- How does the heat shock aid in movement of DNA to the competent cells?
- What is the role of CaCl₂ solution in competent cell preparation?
- How are the competent cells stored for future use?





Experiment 2

Aim Isolation of plasmid. Plasmid alkaline lysis-miniprep protocol.

Key Terminology

- **Chromosomal DNA** The genetic material or the main blueprint of the host organism.
- **Transformed bacterial cell** A bacterium containing an extra plasmid or genetic material of foreign origin.
- **Super-coiled plasmid DNA** “Supercoiled” (or “Covalently Closed-Circular”) DNA is fully intact with both strands uncut, and with a twist built in, resulting in a compact form.
- **Denaturation and precipitation of intracellular protein** The most common observation in the denaturation process is the precipitation or coagulation of the protein, e.g. heat, pH, etc.

Basic Principle

The alkaline lysis method allows for the preferential recovery of circular **plasmid DNA** over linear **chromosomal DNA**. In the alkaline lysis miniprep, developed by Birnboim and Doly (1979) treatment with SDS and NaOH breaks open bacterial cells. Subsequent addition of potassium acetate preferentially reanneals covalently closed plasmid DNA, while chromosomal DNA and proteins are trapped in a complex formed between the potassium and SDS. Subsequent addition of phenol/chloroform leads to denaturation and precipitation of intracellular proteins. The concomitant rapid shrinkage of the cells preferentially expels soluble, super-coiled plasmid DNA into the medium while retaining chromosomal DNA and denatured cellular protein with the bulk of the cell mass. Chromosomal DNA is removed with cellular debris by centrifugation, and soluble, super-coiled plasmid DNA is concentrated by ethanol precipitation.

Materials

- Glucose
- Tris-Cl
- EDTA
- NaOH
- SDS

- Potassium acetate
- Glacial acetic acid
- 3 M sodium acetate
- Phenol
- Chloroform
- Isopropanol
- Isoamyl alcohol
- LB broth (*Annexure III, No. 10 & 11*)
- Double distilled water
- TE buffer (*See Annexure II, No. 2*)
- Eppendorf tubes (1.5 ml)
- Micropipettes
- Tips (200 µl)
- Centrifuge
- Ice
- Ethanol

Chemical Preparation

- **Solution I:** (500 ml) : 4.50 g glucose (50 mm), 1.97 g Tris-Cl (25 mm), 1.86 g EDTA (10 mm). Dissolve reagents in 300 ml dH₂O. Adjust pH to 8.0. Adjust volume to 500 ml. Sterilize by autoclaving.
- **Solution II:** (100 ml): 0.8 g NaOH (0.2 N), 1.0 g SDS (1%). Dissolve reagents in a final volume of 100 ml. Prepare fresh.
- **Solution III:** (100 ml): 29.5 g potassium acetate, 11.5 ml glacial acetic acid. Dissolve reagents in a final volume of 100 ml. pH –4.8. Sterilize by autoclaving.

Methods

1. Pick a single transformed colony and inoculate in 5 ml of LB broth (*See Annexure III, No. 10 & 11*) containing antibiotics e.g., (100 mg/ml ampicillin) and allow the culture to grow at 37°C overnight.
(**Note:** if using naïve non-transformed cells, don't use any antibiotics; simply grow in LB media).
2. Centrifuge 1.5 ml cells in 1.5 ml eppendorf tube at 12000 rpm for 1 minute. Decant the supernatant retaining the bacterial cells pellet. Repeat collection of bacterial pellet by centrifuging 1.5 ml of culture in the same tube if required (preferred for low copy number plasmids).
3. Resuspend cell pellet in 100 µl of solution I (ice cold) and vortex gently.

4. Add 200 μ l of NaOH/SDS lysis solution II. Invert tube 6–8 times slowly.
5. Add 150 μ l of chilled solution III. This solution neutralizes NaOH in the previous lysis step while precipitating the genomic DNA and SDS in an insoluble white, rubbery precipitate. Store on ice for 10 minutes and then spin at 13000 rpm for 10 min.
6. Transfer the supernatant to a new tube. Precipitate the nucleic acids with 0.6 volume of ice-cold isopropanol on ice for 10 min and centrifuge at 13000 rpm for 10 min.
7. Aspirate off all the isopropanol supernatant by decanting, and air dry the pellet. Dissolve the pellet in 100 μ l of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5). Add 5 μ l of RNase A solution (20 mg/ml stock stored at -20°C), vortex and incubate at 37°C for 30 min to digest remaining RNA.
8. Add 100 μ l buffered phenol and mix by inverting and by vortexing 10 seconds.
9. Centrifuge at 12000 rpm at room temperature for 10 minutes. Transfer the top aqueous phase (upper) to new tubes, avoiding interface. Discard organic phase (lower).
10. Add 100 μ l chloroform: isoamyl alcohol (24:1), vortex for 5 seconds to mix. Centrifuge tubes at 13000 rpm for 10 min.
11. Transfer top aqueous phase to new tubes. Add 15 μ l (approximately 1/10 vol. of supernatant) 3 M sodium acetate (pH 5.2). Mix thoroughly by inverting and by vortexing. Place tubes on ice (one may keep the vial in deep freezer for 1 h also).
12. Add 250 μ l (2.5 volumes) of freezer-cold absolute ethanol. Mix by vortexing. Allow DNA to precipitate at -20°C for 3 h or for 30 min at -70°C .
13. Centrifuge tubes at 13000 rpm for 15 min to pellet DNA. Gently pour off the supernatant and discard. Add 250 μ l of 70% ethanol, mix, and centrifuge @ 13000 rpm for 10 min. Discard the supernatant. Air dry the plasmid pellet completely. Resuspend pellet in 30 μ l 1x TE buffer (See Annexure II, No. 2). Store at -20°C .
14. Perform agarose gel electrophoresis to estimate the plasmid size and concentration. (See Experiment 3).

Precautions

- Pure DNA pellets are clear and difficult to observe, therefore, careful handling is necessary to ensure that the product is obtained.
- Grow the culture with appropriate antibiotic for 4–5 h at 37°C in a shaker till the log phase (check for the turbidity of the culture).

Viva Questions



- What is the role of plasmids that naturally occur in a bacterial cell?
- What is meant by alkaline lysis method?
- How can the RNase be made DNase free?
- What is the difference between lysis buffer and TE buffer?
- The method used for the extraction of genomic DNA is not used for the extraction and purification of plasmid DNA. Why?
- What are the essential features required for a plasmid to function as a cloning vehicle or vector?
- Is there any plasmid in eukaryotic cells?



Experiment 3

Aim

Detection of plasmid DNA by agarose gel electrophoresis.

Key Terminology

- Refer Key terminology, Basic principles and Materials required consult Experiment No. 5 of Chapter VI).

Methods

Plasmid DNA is basically present in three different forms, viz., closed circular forms, linear and nicked

forms. Circular forms of DNA migrate in agarose distinctly differently from linear DNAs of the same mass. Typically, uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized. Additionally, most preparations of uncut plasmid contain at least two topologically-different forms of DNA, corresponding to super-coiled forms and nicked circles. Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the $\log_{10}$ of their molecular weight. Therefore, a typical plasmid DNA-agarose gel electrophoresis (see Chapter 1: page 20), in which one set of plasmid DNA has been linearized plasmid, will show a pattern represented by the figure below:

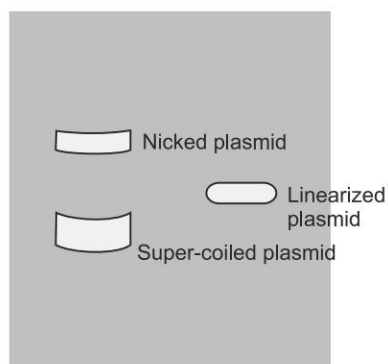


Fig. 7.3.1 Agarose gel electrophoresis showing the migration of nicked and super-coiled plasmid DNA in well 1 and a linearized plasmid DNA in well 2

Observations and Results

For plasmid agarose gel electrophoresis, prepare a 0.8% – 1.5% agarose gel as per the size of the plasmid DNA expected.

Viva Questions



- Why is ethidium bromide used?
- Why do we add dyes to the DNA sample before loading on gel?
- What is a DNA size-marker?
- Name one alternate chemical (other than agarose) that can be used as a gel-matrix for DNA.
- Why is there more than one band in plasmid electrophoresis?
- What is the difference between genomic DNA and plasmid DNA of bacteria?
- Why do DNA bands present in the agarose gel give fluorescence in the presence of UV light?
- Under the same electrophoretic condition, which DNA molecules move faster, larger or smaller.
- DNA molecules move toward which electrode (cathode or anode) in electrophoresis and why?
- Can products of restriction enzyme digestion be isolated by electrophoresis?

Precautions

- Don't pour the gel too hot. Usually it is recommendable to pour the agarose onto the gel plate once it is hot enough that you can afford it to hold with bare hands.
- If you had to boil it for a long time to dissolve the agarose then you may have lost some water to water-vapour. You can weigh the flask before and after heating and add in a little distilled water to make up this lost volume. While the agarose is cooling, prepare the gel tank ready, on a level surface.
- Pour the agarose slowly to reduce the chances of bubble formation.
- EtBr is mutagenic and should be handled with extreme caution. Dispose the contaminated tip into a dedicated EtBr waste container. 10 mg/ml EtBr solution is made up using tablets (to avoid weighing out powder) and is stored at 4°C in the dark with TOXIC label on it.
- People also use TAE (Tris Acetate EDTA). Make up a 50x/10x stock using cheap reagents. Do not use expensive 'analytical grade' reagents. Cheap Tris base and boric acid can be bought in bulk. One must use the same tank buffer as used in making the gel.
- The gel may look set much sooner but running DNA into a gel too soon can give terrible-looking results with smeary diffuse bands.
- Use gel holding scoops to hold the agarose gel after running and transferring it to gel documentation system and disposal etc.



Experiment 4

Aim Restriction digestion of DNA and its analysis by agarose gel electrophoresis.

Key Terminology

- **Restriction enzymes** Enzymes capable of cutting at specific sites in the DNA or nucleic acid molecules depending on its recognition sequence motif's presence on that particular site.
- **Restriction digestion** Chemically chopping or cutting of the nucleic acid material by the restriction enzyme at specific site based on the recognition sequence motif.
- **RFLP** It stands for restriction/fragment/length polymorphism (RFLP) analysis, which is usually applied to screen the restriction digestion pattern of a given DNA or PCR product by using a specific or a combination of restriction digestion followed by gel electrophoresis.
- **One enzyme unit** Is the amount of enzyme required to digest 1 μ g of DNA to complete in 1 h in the recommended buffer and temperature.
- **Sterile nuclease free water** Molecular biology grade water free from DNase and RNase.
- **Palindromic** Is a word, phrase, number or other sequence of units (such as a strand of DNA) that has the property of reading the same either direction (i.e. whether read from 5' to 3' or 3' to 5').
- **Bovine Serum Albumin (BSA)** Also known as "Fraction V" is a serum albumin protein that has numerous biochemical applications. It is a globular protein (66000 dal) with a good essential amino acid profile.

Basic Principle

Restriction enzymes each have their own specific recognition site on double-stranded DNA, usually 6 to 8 bp in length and usually palindromic in sequence. These enzymes allow us to specifically cut DNA into fragments and manipulate them.

Each restriction enzyme has a set of optimal reaction conditions, which are given in the catalogues supplied by the manufacturer. The major variables in the reaction are the temperature of incubation and the composition of the reaction buffer. Most companies supply 10x concentrates of these buffers with the enzymes. These 10x buffers are usually stored at -20°C . Some enzymes also require a non-specific protein. Usually Bovine Serum Albumin (BSA) is used for this and is also supplied as a concentrated solution.

Restriction digestions are performed by incubating DNA molecules with an appropriate amount (units) of restriction enzyme (RE), in its respective buffer as recommended by the enzyme supplier, and at the optimal temperature for that specific restriction enzyme. The optimal sodium chloride concentration in the reaction varies for different enzymes, and a set of standard buffers containing different concentrations of sodium chloride are prepared or supplied and used when necessary.

Typical digestion includes a unit of enzyme per microgram of starting DNA, and **one enzyme unit** is defined as the amount of enzyme needed to completely digest one microgram of double-stranded DNA in one hour at the appropriate temperature. These reactions usually are incubated for 1–3 h, to insure complete digestion, at the optimal temperature for enzyme activity, typically 37°C .

Materials

- DNA/PCR product
- 10X assay buffer/buffer for restriction digestion (See Annexure II, No. 12)
- Sterile nuclease-free water
- Suitable restriction enzyme
- Water bath

Methods

1. Prepare the reaction for restriction digestion by adding the following reagents in the order

listed to a micro-centrifuge tube:

Sterile Double distilled water sufficient quantity to make up to a definite volume

10X assay buffer	one-tenth volume
DNA/PCR product	x μ l
Restriction enzymes*	y μ l–10 units (per μ g DNA)

Total volume z μ l.

An example for setting up a restriction digestion is as follows:

Sterile ddH ₂ O	15.5 μ l
10X assay buffer	2 μ l
DNA/PCR product	2 μ l (~1 μ g DNA)
Hind III Restriction enzymes*	0.5 μ l (~10 units)

Total volume 20 μ l.

2. Gently mix by pipetting and incubate the reaction at the appropriate temperature (typically 37°C) for 3 h in a water bath.
3. Inactivate the enzyme(s) by heating at 72°C for 10 min. An aliquot of the digestion should be assayed by agarose gel electrophoresis versus non-digested DNA and a size marker (e.g. 100 bp marker etc).
4. Follow Experiment 5, to perform agarose gel electrophoresis. Run a gel to confirm the digestion. (See Chapter 1; Page 20)

Result

The undigested (uncut) product size was of _____ base pair. After restriction digestion using the _____ Restriction enzyme overnight, we analyzed the samples on _____ % and found _____

Viva Questions



- What is a specific recognition site of a double-stranded DNA?
- Give examples of some restriction enzymes.
- What do you mean by one unit of restriction?
- What is meant by restriction endonucleases?
- How can the product of restriction digestion be isolated?
- Why are restriction endonucleases very important in genetic engineering?
- What is the use of restriction digest analysis of a DNA sample?
- What is a restriction map of a DNA?

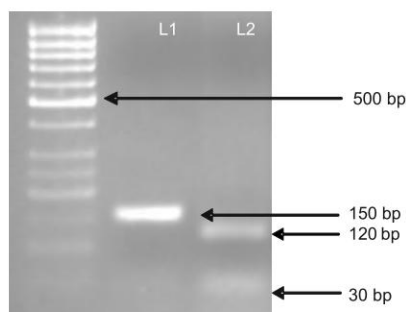


Fig. 7.4.1 Representative figure of a restriction digestion analysis. Lane 1 represents a DNA band of 150 bp; lane 2 represents a restriction digested product of 120 bp and 30 bp. The products are compared with a standard DNA ladder (100 bp).

Precautions

- The volume of the reaction depends on the concentration of the DNA being digested.
- If desired, more than one enzyme can be included in the digest if both enzymes are active in the same buffer and the same incubation temperature (compatible).
- Use sterile pipette tips, for adding each component of the digest to a sterile microfuge tube. Use a clean tip for each addition.
- The order for adding is important. Put water in the tube first, followed by buffer and DNA. Add the enzyme last. Keep digest and enzyme on ice.
- Keep the enzyme back on ice or in a freezer as quickly as possible.
- Mix the content of the tube by tapping with finger, microfuge briefly to bring contents to bottom of the tube.



Experiment 5

Aim **Polymerase chain reaction**—To set up a standard PCR reaction for a specific gene fragment of interest.

Key Terminology

- **PCR or Polymerase chain reaction** which yields in multiple copies of the template with the help of a thermocycler machine using a standard set of chemicals required to initiate and perform the reactions.
- **Taq DNA polymerase** A thermostable DNA polymerase enzyme which catalyzes the synthesis of new nucleic acid template from the 5'→3' end but doesn't have a proof reading activity.
- **Reverse transcriptase** See Expt. 6B, Chapter 6, page 175.

Basic Principle

The **polymerase chain reaction**, usually referred to as **PCR**, is an extremely powerful procedure that allows the **amplification** of a selected DNA sequence in a genome a million fold time or more. PCR can be used to clone a given DNA sequence *in vitro* without the use of living cells during a cloning process. The PCR procedure involves using synthetic oligonucleotides complementary to known sequences to prime enzymatic amplification of the intervening segment of DNA in the tube. The PCR reactions occur in three major steps (called **cycles**), repeated about 25 to 40 times:

1. Denaturing of DNA by high temperatures.
2. Attachment of single-stranded DNA primers that flank the desired DNA at a lower temperature.
3. DNA is synthesized by a special heat-stable DNA polymerase called "**Taq DNA polymerase**" and isolated from the thermophilic bacterium *Thermus aquaticus*.

PCR may be used to

1. Isolate specific sequences for further study.
2. Identify specific genetic loci for diagnostic or medical purposes.

3. Generate DNA fingerprints to determine genetic relationships or in forensics.
4. Rapidly sequence DNA.
5. For converting an unstable mRNA into a stable cDNA sequence utilizing a specific enzyme called **reverse transcriptase** (Also consult Chapter I; i- Polymerase Chain Reaction, Page 17).

Materials

- Sterile nuclease-free water
- 10X amplification buffer (commercially available) (Annexure II, No. 14)
- 15 mM MgCl₂
- 10 mM dNTP
- 10 picomoles/μl oligonucleotide primer 1(Forward primer)
- 10 picomoles/μl oligonucleotide primer 2(Reverse primer)
- 5 Units/μl Taq polymerase
- Template DNA (100 ng genomic DNA, 0.1–1 ng plasmid DNA)

Methods

1. Combine the following for each reaction (on ice) in a 0.2 or 0.5 ml tube:

Sterile water	17.4
10X PCR buffer	2.5 μl
15mM MgCl ₂	1.5 μl
10 pmoles/ μl Primer 1	1 μl
10 pmoles/ μl Primer 2	1 μl
10 mM dNTP	0.5 μl
5U/ μl Taq Polymerase	0.1 μl
Template DNA (100 ng)	1 μl
TOTAL VOLUME	25 μl

2. Prepare a control reaction with no template DNA and an additional 1 μl of sterile water as template.

- Place tubes in a thermal cycler (PCR machine) preheated to 103°C.
- Run the following program:

Steps	Temperature	Time (minutes)	Cycles
Initial denaturation	94°C	2	1
Denaturation	94°C	0.5–1.0	
Annealing (based on T_m^*)	42–65°C	0.5–1.0	25–35
Extension	72°C	1 minute/kilobase	
Final extension	72°C	5	1
Hold	4°C	Indefinite	1

* T_m may be calculated by the formulae $T_m = 2(A+T) + 4(G+C)$ or by considering the datasheet for the given set of primers.

- After completion of the PCR cycle, take out the samples and analyze the results by agarose gel electrophoresis.

Observation

After completion of the PCR and on agarose gel electrophoresis analysis comparing with a standard marker, we got a PCR amplicon of _____ bp.

Viva Questions



- Why is “Taq DNA polymerase” used in PCR reactions?
- What are the different purposes for which PCR can be used?
- What is the function of a thermal cycler?
- Can any DNA polymerase be used in PCR?
- What factors affect the polymerase chain reactions?
- What are amplicons? How are they detected?
- Can PCR assay be used to induce mutations?



Experiment 6

Aim

Gene cloning—To clone a gene of interest in a suitable vector system.

Key Terminology

- Escherichia coli***. See Chapter III; Experiment 5.

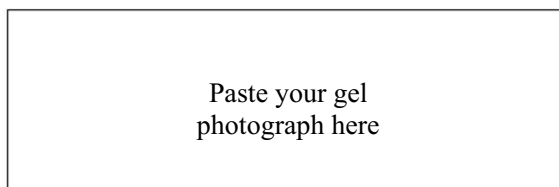


Fig. 7.5.1 Photograph showing a PCR amplified product of _____ bp.

Result

The PCR amplification performed for the _____ gene fragment with specific primers yielded an amplicon of _____ bp on agarose gel electrophoresis.

Precautions

- To perform several parallel reactions, prepare a master mix containing water, buffer, $MgCl_2$, dNTPs, primase and Taq DNA Polymerase in a single tube, which can then be aliquoted into individual tubes.
- Add the desired amount of master mix to the template DNA. This method of setting reactions minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers.
- Gently vortex the sample and briefly centrifuge to collect all drops from the walls of tube.
- Set the conditions in Thermal cycler, place the samples and start PCR.

- Vector** A backbone of extra-chromosomal DNA which can be genetically manipulated to carry the gene fragment of our interest into a reproducible and/or expression system.

- **Ligation** Sticking or ligating two fragments of nucleic acid material with the help of a specialized enzyme called ligase.
- **Gene cloning** The process of synthesizing multiple copies of a particular DNA sequence using a bacterial cell or another organism as a host, e.g. *E. coli*.
- **Expression vectors** Vectors that carry a gene that can be efficiently transcribed and translated by the host cell having regulatory sites.
- **Restriction enzyme digestion** See *Experiment 4*.

Basic Principle

Gene cloning is an integral part of recombinant DNA technology and genetic engineering. This may be achieved using a suitable vector and employing PCR, restriction endonucleases, ligases in different set of reactions. Mostly the region of interest is first amplified by PCR. If PCR amplification of the desired DNA fragment is not possible or desirable, restriction enzyme digestion of the target DNA can be employed. The desired fragment may need to be separated from other DNA fragments in the reaction, so the size of the desired DNA fragment should be known. Once isolated, the fragment is cloned into a vector with compatible ends. If the vector ends are capable of religating (e.g. the vector has blunt ends or is cut with a single restriction enzyme), the vector is often treated with alkaline phosphatase to discourage **recircularization** and maximize **ligation** between the insert and vector.

Following transformation into *E. coli*, the resulting bacterial colonies are screened by PCR for the correct recombinant vector using primers to amplify the insert. Alternatively, the recombinant vector can be identified by performing a **restriction enzyme digestion** to determine the presence of the correct insert or by **direct sequencing** (in case of commercially available vectors like M13 etc). Screening is often simplified by using vectors that contain an antibiotic-resistance gene (See *Experiment 1*), so cells containing the vector will survive on medium supplemented with the appropriate antibiotic. However, vectors

should have **regulatory sites** for the initiation of transcription of its gene and translation of its mRNA by the host cell.

Materials

- Digestion buffer (See *Appendix-Annexure II, No. 20*)
- 2X Ligase buffer (See *Annexure II No.15*). Store in single-use aliquots at -20°C .

Methods

1. Assemble the following reaction components to cut the PCR product/gene of interest with RE1 (e.g. EcoRI) and RE 2 (e.g. Hind III).

Component	Volume
5X Digestion Buffer	4 μl
Purified PCR product (up to 500 ng)	X μl
Enzyme Blend (EcoRI and Hind III)	4 μl

2. Assemble the following reaction components to cut the acceptor Vector with RE1 (e.g. EcoRI) and RE2 (e.g. Hind III).

Component	Volume
Nuclease-Free Water	12 μl
5X Digestion Buffer	4 μl
Vector (200 ng)	2 μl
Enzyme Blend (EcoRI and Hind III)	2 μl

3. Incubate both reactions (Steps 1 and 2) at 37°C for overnight. Heat the reactions at 65°C for 15 min to inactivate the restriction enzymes. Store on ice until the PCR product and vector is put for ligation.
4. Check the product of step 1 by agarose gel electrophoresis, to screen the correct digestion of the PCR components/gene of interest.
5. Cut out the product (size of interest) from the agarose gel, chop, and transfer to a fresh eppendorf tube.

6. Perform the gel extraction process to extract the digested gene fragment of interest using a standard gel extraction kit (e.g., Qiagen gel extraction kit etc) following the manufacturer's protocol.
7. Set out the ligation reaction to incorporate/ligate the gene of interest into the compatible acceptor vector
8. Transform the ligation reaction into high-efficiency, *E. coli* competent cells. Screen at least 5–8 colonies, identified by their ability to grow on both antibiotics.
9. Isolate the plasmid (vector-containing the insert) by following the plasmid extraction protocol, and check the size of the plasmid (which should be equal to the size of vector + insert).

Component	Volume
2X Ligase Buffer	10 μ l
Acceptor Vector—Restriction Digested	2 μ l
Purified PCR product (approximately 100 ng)	X μ l
T4 DNA Ligase (HC; 20 μ g/ μ l)	1 μ l

Precautions

- This experiment should be done only after completing previous (Experiment 1 to 5) or tagging with them.
- Check the list of materials and read the manufacturer's protocol carefully.

Viva Questions



- What components are involved in cloning?
- How is donor DNA obtained for cloning experiments?
- What should be the characteristics of a cloning vector?
- How is the fragment containing a gene of interest identified and isolated for cloning?
- What happens in a cloning experiment after the desired donor DNA is cleaved from the genome with restriction endonuclease?
- What is a vector?
- What are expression vectors?



Experiment 7

Aim **Bacteriophage isolation**—To isolate bacteriophage/coliphages from the sewage sample.

Key Terminology

- **Coliphages** Bacteriophages that use or infect *E. coli* as a host organism are referred to as coliphage.
- **Plaque** A clear zone on a bacterial plate symbolizing the killing/lysis of the bacterial cells resulting in no bacterial growth in that particular zone.

Basic Principle

Bacteriophages are viruses that infect bacteria. Bacteriophages that infect *E. coli* are sometimes referred to as **coliphage**. Generally, bacteriophages are referred to simply as phage, which are host-

specific. Phages must attach to a receptor on the surface of a bacterial cell in order to initiate an infection. Bacteriophage can be found in places where numerous hosts exist. Because coliphage use *E. coli* as a host organism, these phages can be found in places where *E. coli* are plentiful, e.g. in sewage water etc.

Materials

- Tryptic soy broth (See Annexure III, No. 1)
- *E. coli* (isolated from sewage sample earlier/ commercially procured)
- 0.22 micron syringe filter
- Sewage water sample

- Bacterial incubator
- Centrifuge

Methods

Day 1

1. Inoculate 5 ml tryptic soy broth with *E. coli* (isolated from sewage sample earlier/ commercially procured) aseptically. Incubate overnight at 37°C.

Day 2

2. Inoculate another tube with 4.5 ml sewage water, 0.5 ml of your overnight *E. coli* culture, and 0.5 ml of 10 × tryptic soy broths. Incubate 24–48 hours at 37°C.
(During this incubation, phage in the sewage sample capable of binding to your *E. coli* will replicate in and eventually lyse the bacteria.)
3. Centrifuge at 2500 rpm for 10 mins at room temperature to pellet the bacteria and other cell debris at the bottom of the tube.
4. Take up 3 ml of the supernatant into a 5 ml disposable syringe. Attach a 0.22 micron filter to the base of the syringe and filter the supernatant into a sterile tube. (This filter will prevent any remaining bacterial/cell debris (if any) from passing through, but will allow phage to pass through.)

5. Set up another overnight culture of your *E. coli*.

Day 3

6. Transfer and spread 100 µl of an overnight *E. coli* growth onto the centre of a petri-plate containing tryptic soy agar.
7. Next, place 10 µl of your enriched phage prep (filtrate) in the centre of the plate. Incubate at 37°C overnight.

Day 4

8. Examine your plate. (Also consult Chapter III; Experiment 10).

Observation

On day 4, the phage will infect and kill the bacteria in the centre of the plate, creating a visible region of no bacteria—a **plaque**, demonstrating that phage capable of infecting *E. coli* has been successfully isolated.

Result

The resulting free zone in the centre of the *E. coli* plate suggests the isolation of bacteriophages.

Precautions

1. As coliforms, *E. coli* should be carefully handled under supervision.
2. The bacteriophages isolated, and *E. coli* plates should be disposed only after proper autoclaving.

Viva Questions



- Name some single-stranded and double-stranded phages.
- What is transfection?
- What are the differences between lytic and lysogenic life cycle of a phage?
- What does the clear area represent when a phage is speeded over the surface of a bacterial lawn?
- Do all bacteriophages reproduce in the same way? What are the different types of reproduction?
- What do you mean by specificity of phage infection?



Experiment 8

Aim

Transformation by co-cultivation techniques in tobacco plants (leaf discs) using *Agrobacterium tumefaciens*.

Key Terminology

- **Transformation** Introduction of DNA into the cell of an organism by a method other than conventional sexual crossing.
- **Transgenic** An organism in which a foreign gene (a transgene) is incorporated into its genome.

- **vir genes** The *vir* region of the Ti/Ri plasmid is a collection of genes whose collective function is to excise the T-DNA region of the plasmid and promote its transfer and integration into the plant genome.
- **Acetosyrigone** Promotes high efficiency transformation of *Arabidopsis thaliana* or any other plant species explant by *A. tumefaciens*.
- **Co-cultivation** Culturing two or more organisms simultaneously.
- **T-DNA** The segment of DNA in Ti plasmid of *A. tumefaciens* that is transferred to plant cells and inserted into the chromosomes of the plant.
- **Binary vector** A two-plasmid system in *A. tumefaciens* for gene transfer in plant cells. One plasmid contains the virulence gene (responsible for transfer of the T-DNA) and another plasmid contains the T-DNA hoarders, the selectable marker and the DNA to be transferred.
- **Competence** It is the ability of a cell to take up extra-cellular (“naked”) DNA from its environment. Competence may be ‘induced’ or ‘artificial’, arising when cells in laboratory cultures are treated to make them transiently permeable to DNA.
- **GUS-activity** Histochemical staining. The activity of β -glucuronidase (GUS) can be accurately determined in extracts in higher plants.

Basic Principle

A. tumefaciens mediated plant transformation techniques are extensively utilized for developing transgenic plants. The following important requirements are to be fulfilled for transferring gene of interest in higher plants.

1. The plant explants must produce related compounds for activation of *vir* genes or *Agrobacterium* may be pre-induced with synthetic acetosyrigone. The T-DNA transfer to the plant nucleus depends on the expression of the *Agrobacterium vir* genes. For this, the oncogenes are deleted from the T-DNA and

are replaced by suitable marker (antibiotic or herbicide resistant, etc.) gene and gene of interest (new trait to be introduced). The T-DNA construction may be done on another binary-vector, which makes the transformation process more adapted to many requirements. The integration of T-DNA depends on the plant DNA repairing machinery. Therefore, one copy of the T-DNA is inserted randomly in the plant genome (preferably in the vicinity of transcribed region).

2. The induced agrobacteria should have access to cells that are competent for transformation.
3. Transformation competent cells or tissues should be able to regenerate into whole plants.

The explant used for inoculation (or **cocultivation**) with *Agrobacterium* carrying the desired **binary vector** include cells, protoplasts, callus, leaf discs, tissues, whole organs or sections, etc. The following three approaches are available for inoculation of *Agrobacterium*.

- (a) **Infection of wounded plants:** Well established *in vitro* plants, under aseptic conditions are inoculated to obtain *Agrobacterium* transformed cells. *In vivo* seedlings are decapitated and the freshly cut surface wound is inoculated with an overnight culture of *Agrobacterium*. The tumor produced is excised and grown as a callus culture. The transformed calluses are collected and regenerated.
- (b) **Co-cultivation:** Protoplasts are isolated and at the time of cell wall reformation, these cells are co-cultivated along with *Agrobacterium* (at about 100 bacteria per protoplast cell) and incubated for 24 to 40 h in a suspension culture. Transformation occurs during few days of co-cultivation. Transformed cells can be selected using selective agent.
- (c) **Leaf disc method:** Any tissue explant that provides a good source for initiation of whole plant differentiation (e.g. newly emerged cotyledons) is selected and allowed

to incubate in *Agrobacterium* suspension for few hours to three days and then cultured on a media for bacterial growth to take place. Tissue explants (viz. discs) are then transformed to a media containing antibiotics to eliminate the bacteria. Transformed cells are isolated using selective agent. The isolated discs are cultured in an appropriate medium for regeneration.

The disc method is technically simple and can be carried out in an ordinary Plant Tissue Culture laboratory for producing transgenic plants very rapidly. Therefore, protocol for this method will be described in this experiment. If necessary consult related experiments in *Chapter IV*.

Materials

- Plant material—Leaf discs of *Nicotiana tabacum*.
- Bacteria strain—LBA 4404 (p^{BI121}). The binary vector p^{BI121} contains *gus* reporter gene and neomycin phosphor-transferase conferring resistance to Kanamycin as a selected marker.
- *A. tumefaciens* culture in glycerol stock.
- YEP medium (with and without antibiotics, viz. Kanamycin, Streptomycin and Refampicin (See *Annexure III; Sl. No. 18*))
- MS medium (With BAP and NAA) (See *Annexure IV*)
- Gus substrate (For composition see *Page 200*)
- Sterilized petri-plates, conical flasks
- Membrane filter
- Inoculating needle
- Rotary shaker
- Spectrophotometer
- Incubator maintained at 28°C
- Sterile Whatmann filter paper
- Sterilized distilled water

Methods

1. Prepare *Agrobacterium* culture. Take an appropriate strain containing binary vector p^{BI121} , containing Kanamycin, Streptomycin and Refampicin as bacterial selectable marker genes.
2. Prepare YEP medium without and with antibiotics (See *Annexure III; Sl. No. 18*).
3. Inoculate *Agrobacterium* strain from Glycerol stock on a petri-dish using a sterile inoculation needle by streaking method (See *Chapter III; Expt. 1*). Incubate at 28°C.
4. Next day pick a single colony from the petri-dish and inoculate into 23–20 ml of YEP liquid medium containing in a 100–230 ml flask. Incubate on a rotary shaker with 120 rpm speed at 28°C.
5. Prepare the following MS₁ medium for tobacco leaf discs culture. MS + 1.0 mg/l BAP + 0.1 mg/l NAA. (See *Chapter IV; Expt. 2*).
6. Collect young, healthy green leaves from tobacco plants to be transformed. Cut into small pieces avoiding the mid-vein portion.
7. Bring it to the laminar air-flow unit and transfer them into a beaker containing sodium hypochlorite (1–2% active chlorine) for 10 min.
8. Wash the leaf samples 3 to 4 times with sterile distilled water. Cut the leaf into small discs of 1 cm diameter.
9. Take the overnight grown culture of *Agrobacterium* and measure the OD at 600 nm. For co-cultivation, centrifuge the *Agrobacterium* culture at 2500 rpm for 23–20 min. Discard the supernatant and resuspend the bacterial pellet in prepared MS medium to bring the OD to 0.1.
10. Transfer the *Agrobacterium* suspension into a sterile petri-plate and put the cut sterile leaf discs in the *Agrobacterium* suspension for 30 min.
11. Take the leaf discs out after 30 min and dry them on a sterile Whatmann filter paper.
12. Inoculate these leaf discs on MS (with supplementation) medium. Incubate for 3 days at 16 h photoperiod.
13. After 3 days, transfer the discs individually to MS₂ medium, containing bacterio-static agent (MS₁ + 300 mg/l Carbenicillin or Cefotaxime) and culture again under the same conditions.

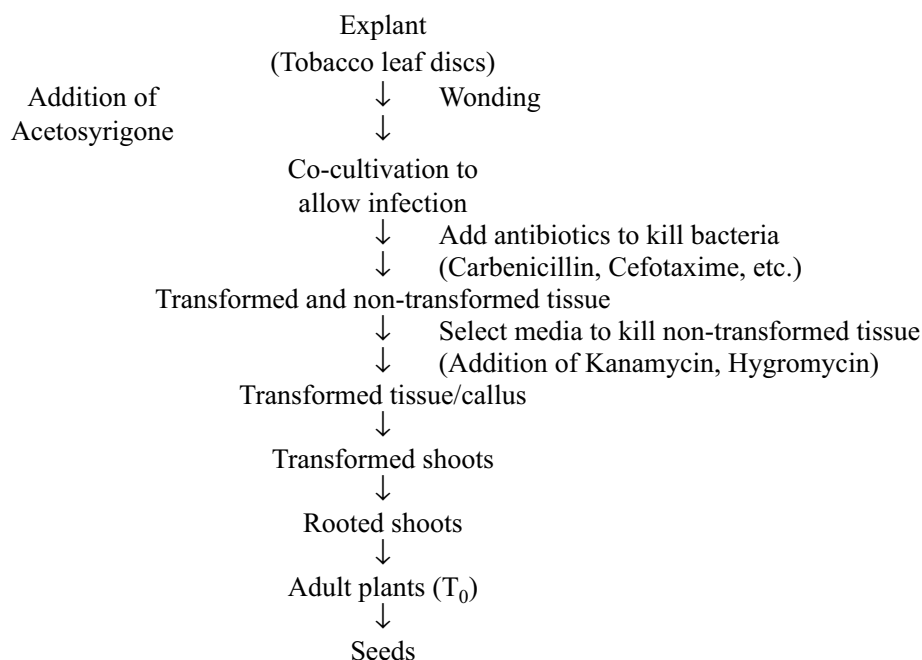


Fig. 7.8.1 Steps for *A. tumefaciens* mediated transformation of explants

14. After 3 days, transfer the leaf discs individually to MS₃ medium, containing selective agent as per the selectable marker used in transformation experiments (e.g. for p^{BI121} – MS₂ + 100 mg/l Kanamycin). Culture under same condition.
15. Take out the samples from time to time or select the regenerating transformed shoots and assay for GUS activity (*See below*).
16. Wash the explants with distilled water and acclimatize the plants in field or pots.
3. Before first use, add 60 µl of Triton-X for each 100 ml.

Observations

Any of the following methods may be employed for selection of transformed plants:

1. Incubate few explants in GUS substrate (inoculate overnight in the dark at 37°C for 36 h) after the period watch for transient GUS expression. The endogenous GUS activity will be shown by non-transformed (normal) explants.

OR

2. Place the explant material in wells of ELISA plates with X-Gluc strain till it is completely submerged. Incubate the plates at 37°C for 36 h. Wash and keep it stained in 95% ethanol for preservation.

OR

3. The explants may also be tested for selection of transformed plants using appropriate selection agent (antibiotics or herbicides, etc.) depending on the plant selectable marker gene employed for transformation. Resistant/

Preparation for GUS activity:

* K ⁺ ferricyanide	– 0.4 mM
* K ⁺ ferrocyanide	– 0.4 mM
Sodium phosphate buffer (pH 7.0)	– 80 mM
(See Annexure –II; Sl. No.16(a) or (b))	
EDTA	– 8 mM
Triton X-100	– 0.05%
5 bromo-4-chloro-3 indolyl-β-D glucuronide	– 0.8 mg/ml

* See Precautions

1. Filter sterilizers using 0.2 micro filters.
2. For 100 ml of GUS staining solution, add 25 ml MeOH (to give 20% MeOH concentration).

tolerant explants are continuously grown and selected followed by regeneration.

OR

- Transgenic plants may also be tested for stable integration and expression of genes by PCR method.

Result

Presence of blue spots indicates *gus*-gene expression. The regenerated plants will be T_0 transgenic plants and seeds obtained from them will be T_0 seeds. Plants obtained from these T_0 seeds will be T_1 plants.

Look for the character expression of gene of interest which has been transferred.

Precautions

- While handling K^+ ferri- and ferro-cyanide be careful as these are **highly poisonous**. As such, top precautions should be taken during

handling. Hand gloves must be used while handling these chemicals.

- Before commencement of the experiment appropriate *Agrobacterium* strain and antibiotics which are capable of controlling bacteria should be selected.
- A. tumefaciens* strain that is capable of transferring part of its plasmid DNA (called T-DNA), encoding oncogenes and will induce the tumour on the infected plant tissue should be selected.
- YEP suspension culture, MS_1 medium and GUS stain (after adding BAP and NAA) and GUS stain should be membrane filtered using 0.2 micro filters.
- Antibiotics are to be added after cooling down the autoclaved medium to $50^\circ C$.
- GUS activity at the cut ends indicates the susceptibility of explants to *Agrobacterium* mediated transformation.

Viva Questions



- Do eukaryotic controlling sites differ from those in bacteria?
- What role does the structure of chromosome play in controlling DNA replication and transcription?
- What are the advantages of *Agrobacterium* mediated transformation?
- Name some other vectors that are used for transformation.
- What characteristics should a useful vector possess?
- What are the other methods of DNA transfer?
- Why is GUS activity necessary? What does it reveal?



Experiment 9

Aim

Medical Biotechnology—To separate blood mononuclear cells by Ficoll hypaque and observing viable PBMC's by trypan blue staining.

Key Terminology

- Lymphocytes** It was identified as the cell responsible for both cellular and humoral immunity. They are of two basic types, viz. T lymphocytes and B lymphocytes.
- PBMC/PBMNC** Peripheral blood mononuclear cell.
- Metrizoate** 3 acetamide—2:6:6 tri-iodo-N methyl acetamidobenzoate.

Basic Principle

Trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live cells do not take up certain dyes, whereas dead cells do.

Materials

- Trypan blue (0.4% suspension)
- Hemocytometer
- Slide and cover slip
- Microscope

Methods**Separation of Mononuclear Cells**

1. Take 15 ml of blood and dilute it with phosphate buffer saline/RPMI 1640 media.
2. Take 10 ml of Ficoll in another falcon and layer the diluted blood over it very carefully.
3. Spin it for 30 min at 1500 rpm (preferably using swing out rotors).
4. Take out the middle layer of peripheral blood mononuclear cells with the tissue culture pipette and transfer into another falcon.
5. Spin the PBMC's for 10 min at 1500–1600 rpm.
6. Wash them with RPMI 1640/PBS – 10 ml and spin for 15 min at 1500–1600 rpm. Decant the supernatant.
7. Repeat step-6 twice.
8. Add freezing media (90% FCS and 10% DMSO)— 2 ml in PBMC's and freeze them in -70°C or in liquid nitrogen.
9. Combine 20 μl of cells with 20 μl of trypan blue suspension (0.4%). Mix thoroughly and allow it to stand for 15 min.
10. With the cover slip in place, transfer a small amount of trypan blue-cell suspension to both chambers of the hemocytometer by carefully touching the edge of the cover slip with the pipette tip and allowing each chamber to fill by capillary action.
11. Starting with 1 chamber of the hemocytometer, count all the cells in the 1 mm centre square and four 1 mm corner square. Keep a separate count of viable and non-viable cells.

Viva Questions

- For what are blood lymphocytes responsible?
- What are the two basic types of lymphocytes?
- How is PBMC cell suspension prepared?

**Experiment 10A****Aim**

Bioinformatics. To analyze a given gene sequence for mutation by software analysis.

Key Terminology

- **Bio-informatics** A branch of science which does the networking between computer/

electronics and biological sciences. Bioinformatics is also a part of the dry lab

12. If there are too many or too few cells to count, repeat the procedure either concentrating or diluting the original suspension as appropriate.
13. The circle indicates the approximate area covered at 100X microscope magnification (10X Ocular and 10X Objective).

Observations

1. Include cells on top and left touching middle line. Do not count the cells touching the middle line at the bottom and right. Count 4 corner squares and middle square in both chambers and calculate the average.
2. Each large square of the hemocytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3 . Since 1 cm^3 is equivalent to approximately 1 ml, the total number of cells per ml will be determined using the following calculations:

$$\text{Cells/ml} = \text{Average cell count per square} \times \text{dilution factor} \times 10^4$$

Result

Total cells = Cells/ml $\times$ Original volume of fluid from which the cell sample was removed; % Cell viability = Total viable cells (unstained)/total cells $\times 100$.

Precautions

- In Methods: Step 2, do not overfill or underfill the chamber of hemocytometer.
- Adjust your observation depending on the frequency of viable (unstained) and dead (stained) cells in both chambers of the hemocytometer.

constituent in Biotechnology. Bioinformatics helps in multiple ways to predict and solve queries related to structural biology, homology search, conformational analysis, mutational research, drug designing, molecular modelling and to figure out novel therapeutic targets for drugs under investigation.

- **Databank** A virtual organization which stores the analyzed, proved and reported data thereby making them available for other intended users and researchers to utilize the data stored in these databanks as comparative standards in their work or analysis.
- **Mutation or Polymorphism** A change in nucleotide sequence prevalent in less than 1% of the population.
- **Electrophorogram** Is a plot of results from an analysis done by electrophoresis which is automatic and used in interpretation in genomic sequencing project.

Basic Principle

Mutation or polymorphism results when there is a change in sequence of the nucleotide, thereby resulting in changes in amino acid sequences in many cases which may in turn change the functionality of a protein, expression of a protein, or abrogation or short termination of the protein as the case may be depending on the change as well as the place (promoter, non-coding or coding region) where the change is taking place. The sequences of majority of the genes of the organisms have been delineated by researchers all over the world and have been maintained in distinct data bases such as National Center for Biotechnology Information (NCBI), Data Bank of Japan (DDBJ) and European Molecular Biology Laboratory in Europe (EMBL), etc. All of these data bases are cooperative systems. The standard sequences for a specific gene of a specific organism serve as a template for comparison while analyzing any sequence variants (mutation or polymorphism) using different available software's. The whole

platform of Biotechnology is divided into two parts, i.e. wet lab and dry lab. The dry lab constitutes the Computational Biology and Bioinformatics which utilizes and analyzes the data obtained by the wet lab with the help of computer, graphics and softwares.

Materials

A computer with a few basic softwares and internet connectivity.

Methods

1. In the very beginning, perform a PCR for a specific gene or gene fragment of interest with specific primers. Purify the PCR product by agarose gel electrophoresis followed by gel extraction or PCR purification protocol using standard commercially available kits following the manufacturer's protocol. Send the purified product for direct sequencing in a commercially sequencing centre along with 5 pmoles/ μ l of each forward and/or reverse primers. The sequencing result will generate an **electrophorogram** and a word document of the sequence which will be made available to you for further analysis. The peaks for each nucleotide are designated by different colours. An example is shown in Fig. 7.10A.1.
2. The electrophorogram can be opened, read and corrected for any 'N' if present or mistake by the sequence reading software already installed along with the sequencing machine (in case of heterozygosity cases etc.), in a computer system using softwares like Bioedit, Generunner, Finch TV, etc. This may be also done manually if the softwares are not available with the user.
3. After correction (if any) and checking the electrophorogram and nucleotide sequence provided as an output of the direct sequencing, one may now go for the comparison of the sequences against the standard GenBank data from databases for the specific gene of

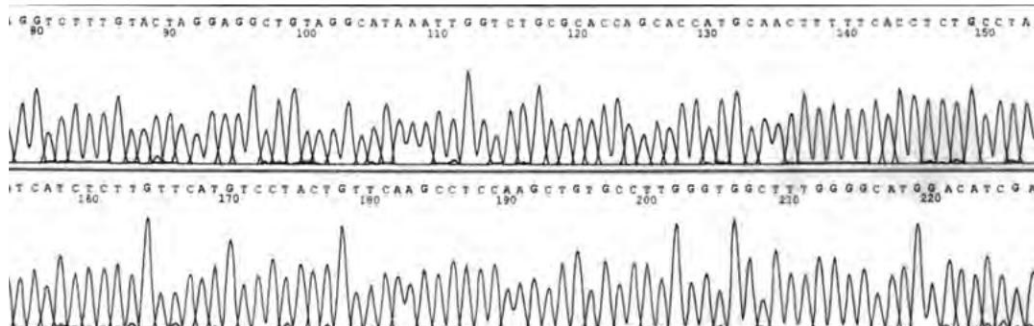


Fig. 7.10A.1

interest under analysis, following the steps mentioned below:

- Retrieve or download the standard sequence of the gene under analysis from the standard data banks like NCBI (<http://www.ncbi.nlm.nih.gov>) or ensemble (<http://www.ensembl.org>).
- If you have your sequence and standard sequence ready with you, you may now go for comparative analysis directly from the NCBI website through the choices BLAST → pairing of two nucleotide sequences → align, and check for any mispairing against the standard sequence which indicates the change in nucleotide (mutation or polymorphism) in your sequence. On the other hand, if you have multiple (more than two) sequences needed to be compared, then you may perform the analysis using freely available softwares ‘Multalin’ or ‘Clustal W’, etc. For instance, simply go to the Multalin software tool website through the common search engines like **Google** via the internet. The window in the Multalin software requires you to name and input your sequences, (e.g. see Fig. 7.10A.2)

```
>Seq1xyz
TGCGCACC.....T....
>seq2xyz
TGCGCACC.....T....
>Seq3xyz
TGCGCACC.....A.....
>Seq4xyz
TGCGCACC.....A.....
>Seq5xyz
TGCGCACC.....T.....
>Stdxyz
TGCGCACC.....T....
```

Fig. 7.10A.2

There are few other parameters given below this window that needs to be modified or left alone as per your need, and after finishing with all the inputs, click the ‘Submit’ button provided at the bottom. Allow the software to run the alignment programme automatically and will finally provide you with an aligned sequence windows showing homology in sequence or alteration in nucleotide sequence in different colours as shown in Fig. 7.10A.3.

```
Seq 1xyz TGCGCACCAGCACCATGCAACTTTTTACCTCTGCCTAATCATCTCTTGTTCATGTCCTACTG
Seq 2xyz TGCGCACCAGCACCATGCAACTTTTTACCTCTGCCTAATCATCTCTTGTTCATGTCCTACTG
Seq 3xyz TGCGCACCAGCACCATGCAACTTTTTACCTCTGCCTAATCATCTCTTGTACATGTCCTACTG
Seq 4xyz TGCGCACCAGCACCATGCAACTTTTTACCTCTGCCTAATCATCTCTTGTACATGTCCTACTG
Seq 5xyz TGCGCACCAGCACCATGCAACTTTTTACCTCTGCCTAATCATCTCTTGTTCATGTCCTACTG
Stdxyz  TGCGCACCAGCACCATGCAACTTTTTACCTCTGCCTAATCATCTCTTGTTCATGTCCTACTG
Consensus seq TGCGCACCAGCACCATGCAACTTTTTACCTCTGCCTAATCATCTCTTGTTCATGTCCTACTG
```

Fig. 7.10A.3

Observation

The analysis of the aligned sequences against the comparative standard gene bank sequences reveals the presence of mutation $T \rightarrow A$ and $T \rightarrow C$ in samples 3 and 4.

Result

Out of the five sequences analyzed, 2 samples, i.e. sample 3 and 4 harboured mutation at two positions.

**Experiment IOB**

Aim To analyze a given protein sequence for mutation by software analysis.

Key Terminology

- **Protein** A functional translated product of a gene expressed in a cellular system.
- **Software** A computer-based program which helps in delineating a result for a given set of problem(s).

Basic Principle

Changes in nucleotide sequences often result in changes of the amino acid sequence which is critical for the functioning of any given protein. Analysis of these changes is crucial and can be performed by using Bioinformatics software tools.

Materials

A computer with a few basic softwares and internet connectivity.

Methods

1. Open the internet, and through the Google search engine go to the website for 'Expasy'. The Expasy tools provide you with multiple facilities to perform a wide range of applications. Choose the option which indicates application to perform translation from nucleotide sequence to amino acid sequence.

2. Input the nucleotide sequence and the program will translate it to amino acid sequence. Repeat the same for getting the amino acid sequence of multiple nucleotide sequences including the standard sequence for the gene investigated.
3. Align the sequences using the 'Multalin software' as done in the earlier experiment. The window after completion of the run will show the aligned amino acid arrangement, as shown in the example below.

```
Seq 1xyz FGASVELLSFLPSDFFPS IRDLLDTASAI
Seq2xyz FGATVELLSFLPSDFFPS VRDLLDTASAI
Stdxyz   FGATVELLSFLPSDFFPS VRDLLDTASAI
Consensus seq FGATVELLSFLPSDFFPS VRDLLDTASAI
```

4. The variation in amino acid sequences (due to mutation in the original nucleotide sequence) will be represented by a different colour compared to the homologous amino acid sequences which will be represented by a different colour set of choice.

Observation and Result

The amino acid alignment shows changes in sample 1 (seq1xyz) at two positions compared to the other 2 samples (seq2xyz and seq3xyz) and the standard gene bank sequence for the gene. This may result in altered functionality of the protein coded for by gene under investigation.

Notes and Sketches





8

Animal Biotechnology and Immunology



Experiment I

Aim To learn the basics of animal cell culture.

Key Terminology

- **Mycoplasma** A group of small parasitic bacteria that lack cell walls and can survive without oxygen, can cause pneumonia and other diseases.
- **Aerosols** It is a suspension of fine solid particles or liquid droplets in a gas, e.g. oceanic haze, air pollution, smog, etc.
- **Antimycotic** Antifungal. Any agent that destroys or prevents the growth of fungi.
- **RPMI 1640/DMEM** Patented medium for cell culture. RPMI-Royal Perk Memorial Institute (*culture medium*) and DMEM stands for Dulbecco's Modified Eagle's Medium (*for cell culture growth*).
- **DMSO** Dimethyl sulfoxide $[(CH_3)_2SO]$, is a colourless hygroscopic liquid chemical compound obtained from legnin.
- **Fetal Bovin Serum (FBS)** Also known as Foetal calf serum. It is the portion of plasma remaining after coagulation of blood, during which the plasma protein fibrinogen is converted to fibrin and remain behind in the clot.

Recommendations

1. Use personal protective equipment (laboratory coat/gown, gloves, etc.) at all times.
2. Keep all work surfaces free of waste.
3. Correctly label reagents including flasks, medium with contents and date of preparation.

4. Only handle one cell line at a time to reduce the possibility of cross-contamination. It will also reduce the spread of bacteria and mycoplasmas by the generation of **aerosols** across numerous opened media bottles and flasks in the working cabinet.
5. Clean the work surfaces with a suitable disinfectant (e.g. 70% ethanol) between operations and allow a minimum of 15 min between handling different cell lines.
6. Examine cultures and media daily for evidence of gross bacterial or fungal contamination. This includes medium that has been purchased commercially.
7. Ensure that incubators, cabinet, centrifuges and microscopes are cleaned and serviced at regular intervals.

'Avoid'

1. Don't allow waste to accumulate particularly within the laminar cabinet or in the incubators.
2. Don't handle cells from unauthenticated sources in the main cell culture suite.
3. Avoid keeping cell lines continually in culture without returning to frozen stock.
4. Always sub-culture at 70–80% confluency.
5. Do not allow media to go out of date.
6. Avoid water baths from becoming dirty and don't allow essential equipment to become out of calibration. Ensure safety cabinets are tested regularly.

Preparation of Cell Culture Media and Solutions

Antimycotic/antibiotic Media

To 1 litre of sterile RPMI 1640/DMEM with 2 mM L-glutamine, add, 165.0 ml Foetal bovine serum (heat inactivated), 12.0 ml 200 mM (100X) L-glutamine, 12.0 ml antimycotic/antibiotic (100X) liquid.

Filter sterilize through a 0.22 μ m cellulose acetate filter and store up to 2 weeks at 4 °C.

Freezing Media (1 litre)

Store the tubes at –80 °C for up to one year.

700 ml RPMI-1640 with 2 mM L-Glutamine

200 ml foetal bovine serum (FBS)

100 ml dimethyl sulfoxide (DMSO)

Note: Filter sterilize media and FBS with a 0.22 μ m cellulose acetate filter. DO NOT filter DMSO (it will dissolve the cellulose acetate membrane).

Growth Media (1 litre)

To 1 litre of sterile DMEM/RPMI 1640 with 2 mM L-glutamine, add:

165.0 ml foetal bovine serum (heat inactivated)

12.0 ml 200 mM (100 X) L-glutamine

1.2 ml 50 mg/ml gentamicin reagent

Note: Filter sterilize through a 0.22 μ m filter and store up to 2 weeks at 4°C.

Wash Media (1 litre)

To 1 litre of sterile DMEM/RPMI 1640 with 2 mM L-glutamine, add:

10.0 ml 2.5 M (100X) Hepes buffer (*See Annexure II; No. 7*)

1.2 ml 50 mg/ml gentamicin reagent

Note: Filter sterilize through a 0.22 μ m cellulose acetate filter and store up to 2 weeks at 4°C.

The composition and requirement of media to be used depends on the cell line under culture.



Experiment 2

Aim To describe the maintenance of cells in culture.

Key Terminology

- **Confluent culture** A cell culture in which all the cells are in contact and almost the entire surface of the culture vessel is covered. It is also often used with the implication that the cell have reached their maximum density, though confluence does not necessarily mean that division will cease or that the population will not increase in size.
- **Cellular 'glue'** Protein molecules that are imbedded in the cell membrane of animal cell. It gives tissues their shape and helps keep them strong.
- **Proteolytic enzyme** *See Chapter V ; Experiment 10*
- **Trypsin (or collagenase)** Two protease enzymes e.g. Trypsin and collagenase. Used in mechanical harvesting on cell membrane lipids in monolayer culture of animal cells.

Basic Principle

Most animal cell lines and primary cultures grow as a **single thickness cell layer** or sheet attached to a plastic or glass substrate. Once the available substrate surface is covered by cells (a **confluent culture**), growth slows and then ceases. Therefore, in order to keep the cells healthy and actively growing, it is **necessary to subculture them** at regular intervals. Usually, this sub-cultivation process involves **breaking the bonds** or **cellular 'glue'** that attaches the cells to the substrate and to each other by using **proteolytic enzymes** such as **trypsin** or **collagenase**. The loosened cells are then removed from the culture vessel, counted, diluted and subdivided into new vessels. Cells then reattach, begin to grow and divide, and, after a suitable incubation period (depending on the initial inoculum size, growth conditions and cell line), again reach saturation or confluency, after which the sub-cultivation cycle has to be repeated or the cells needs to be **frozen** till further use.

Materials

- Complete cell culture medium, appropriate for the cell line.
- Tissue culture flasks of appropriate sizes
- Sterile pipettes (assorted sizes).
- Sterile tips.
- Pasteur pipettes, sterile, unplugged.
- 70% alcohol.
- 0.1% Trypsin solution: Trypsin is normally used in concentrations ranging from 0.05% to 0.25%. Working concentrations are usually determined by using the lowest trypsin concentration that can remove the cells from the substrate and give a single cell suspension in a relatively short time (5 to 10 min). Trypsin solutions are often supplemented with other enzymes (collagenase) or chelating agents (EDTA) to improve its performance.
- Calcium- and Magnesium-Free Phosphate-Buffered Saline (CMF-PBS).

Equipments

- Laminar flow hood
- CO₂ Incubator
- Haemocytometer
- Mechanical pipetter (Pipetman)
- Inverted microscope
- Vacuum pump and flask
- Water bath maintained at 37°C

Methods**A. Sub-culturing of Non-adherent Suspension Culture**

1. Turn on the Laminar flow hood and allow to run for at least 10 min before starting.
2. Pre-warm all media in 37°C water bath.
3. Wipe all surfaces with 70% alcohol before starting.
4. Remove the flasks from the incubator. Examine flasks with an inverted microscope.
5. Place flasks and medium under hood.
6. Remove an aliquot of the cell suspension for counting with a hemocytometer and viability determination (*by trypan blue viability test—*

see Experiment 3).

7. Label the appropriate number of new flasks with the cell line name, the passage, the slit ratio or seeding density and the date.
8. Aseptically transfer the required number of cells to the new flask. Add fresh medium to the flask.
 - For T-25 flask—4 ml to a maximum of 10 ml of medium.
 - For T-75 flask—15 ml to a maximum of 50 ml of medium.
9. Recap the flask(s), gently shake to evenly disperse the cells.
10. Return the flasks to the CO₂ incubator. Loosen the caps 1/2 turns (if necessary).

B. Sub-culturing of Adherent Culture

1. Turn on the hood and allow to run for at least 10 min before starting.
2. Pre-warm all media in 37°C water bath.
3. Wipe all surfaces with 70% alcohol before starting.
4. Remove the flasks from the incubator. Examine flasks with inverted microscope.
5. Place flasks and medium under hood.
6. Using a sterile pipette, remove and discard the culture medium.
7. For a T-25 flask, wash the cell monolayer by adding 2 to 4 ml of CMF-PBS to the flask and then slowly rock it back and forth to remove all traces of foetal bovine serum. Remove and discard the wash solution.
8. Add 2 ml of the trypsin solution (in CMF-PBS) to the flask and place the flask back in an incubator at 37°C to increase the activity of the enzyme solution for 2–5 min (gently tap the side of the flask to detach cells from the plastic surface and check the dissociation of cells under the inverted microscope).
9. Add 5 ml of growth medium to the cell suspension and, using a 5 ml pipette, vigorously wash any remaining cells from the bottom of the culture vessel.
10. Collect the suspended cells in a 15 ml centrifuge tube (or 1.5 ml eppendorf tubes) spin the cell suspension at 1000–2000

rpm for 5 min. Then remove the trypsin containing medium and replace with fresh medium (containing serum).

11. Remove an aliquot of the cell suspension for counting with a haemocytometer and viability determination.

Table 8.2.1 Predicted cell yields and recommended medium volumes for few flasks

CULTURE FLASK SIZES	AVERAGE CELL YIELD*	RECOMMENDED MEDIUM VOLUME (ML)
T-25 (25 cm ²)	2.5×10^6	4–7.5
T-75 (75 cm ²)	7.5×10^6	15–22.5
T-150 (150 cm ²)	1.5×10^7	30–45

* Cell yield may vary depending on the cell line used.

Note: Most mammalian cell cultures do best at a temperature between 35° and 37°C. In addition to maintaining constant temperature, CO₂ concentrations (usually 5% to 10%, depending on bicarbonate concentrations in the medium) help maintain the proper pH (7.4 ± 0.2) to avoid stress on the cells in culture. Examine cultures daily and change medium as needed.

Viva Questions



- What is a confluent culture? Why is it necessary to sub-culture them?
- Why are proteolytic enzymes used? Give examples of a few proteolytic enzymes.
- What are the differences between non-adherent and adherent suspension cultures?



Experiment 3

Aim To estimate the number of viable cells using the trypan blue viability test method.

Basic Principle

Trypan blue will stain dead or dying cells. Viable cells are able to repel the dye and do not stain (See Chapter VII; Experiment 9A).

Materials

- Eppendorf tubes (1.5 ml)
- Micropipet (10 µl)
- Hemocytometer
- Sterile Trypan blue solution 0.4%

12. Label the appropriate number of new flasks with the cell line name, the passage, the slit ratio or seeding density and the date.
13. Aseptically transfer the required number of cells to the new flask. Add fresh medium to the flask.
14. Recap the flask(s), gently shake to evenly disperse the cells.
15. Return the flasks to the incubator. Loosen the caps 1/2 turns (if necessary).

Results

Continuity of culture growth is an indication of viable cells. The total percentage of viable cells in the culture was found to be _____ %.

Precautions

- Add fresh growing medium at the time of sub-culturing of non-adherent and adherent cultures.
- Use medium volume (in ml) according to the size of the culture and average cell yield.
- Keep close watch on the culture every day.

Methods

1. Prepare a cell suspension in cell culture media.
2. Transfer into an Eppendorf tube:
 - 0.5 ml of 0.4% Trypan blue solution
 - 0.3 ml of cell culture media
 - 0.2 ml of cell suspension in cell culture media (dilution 1 : 5)
3. Allow to stand for 5 to 15 minutes.

4. Pipet 1 μ l of this mixture into cover-slipped chambers of the haemocytometer.
5. Count the viable and non-viable cells.

Note: For optimal results, adjust cell density to 20–50 cells / square.

Observation and Calculations

- **cells/ml:** the number of cells per quadrant equals 10^4 cells / ml (e.g. 20 cells per quadrant = 0.20 million cells/ml)

- **total cells:** cells / ml $\times$ original volume (e.g. 2 million cells in 10 ml)
- **cell viability (%):** total viable cells (unstained)/total cells (stained and unstained) $\times$ 100 (e.g. 10 stained cells per quadrant: 50% viability)

Results

The total percentage of viable cells in the given culture was found to be _____ %.



Experiment 4

Aim Protein extraction and estimation from cell lines by immuno-blotting.

Key Terminology

- **Immuno-blotting (or Western blotting)** Is an analytical technique used to detect specific proteins in a given sample of tissue homogenate.
- **Chemiluminescence immunoassay** Is now established as one of the best alternatives to conventional radio-immunoassay for the quantitation of low concentrations of analysis in complex samples.
- **Differential expression** A multifaceted approach used in which differential protein expression can be studied e.g. by two-dimensional (2D) gel electrophoresis.

Basic Principle

Immuno-blotting or Western blotting is a prescribed technique to study the differential expression of proteins. The technique involves multiple steps: extraction and estimation of the total protein from the source material (tissue/cell lines etc.), separation of total proteins on a poly-acrylamide gel (SDS-PAGE) (See Chapter VI; Experiment 8 and also Chapter I; j. Electrophoresis), transfer of the separated proteins from the poly-acrylamide gel to a nitrocellulose/nylon membrane, specific antibody hybridization targeted against a specific protein and subsequent detection using a suitable detection technique (e.g. **chemiluminescence** based method). The final detection results may

then be analyzed manually or using densitometry. The end result will reveal the differential expression of a target protein in compared samples.

Materials

- Cell line
- MLB protein extraction buffer (*Annexure II, No. 8*)
- BCA kit
- Bovine serum albumin (instead fat free milk may also be used)
- Phosphate buffer saline (See *Annexure II, No. 16*), with 0.1% Tween-20 added (PBST)
- Transfer buffer (1.5 l) (See *Annexure II, No. 17*)
- Chemiluminescence Western blotting developing kit
- Eppendorf tubes
- Western blotting apparatus with cassettes
- Power pack
- X-ray film
- Primary antibody
- Secondary antibody-HRP labelled and specific to the primary antibody (e.g. anti-mouse-HRP for primary antibody developed in mouse)
- Whatmann filter paper
- Nitrocellulose/nylon membrane
- Suranwrap
- Developer

- Fixer
- SDS-PAGE and running apparatus

Methods

A. Extraction of Total Protein and Quantification

1. After incubation of 3 days the cell culture, observe for 80–90% confluence.
2. Take out the media and wash with tissue culture grade PBS.
3. After washing, add 1 ml of PBS to each flask containing the cell lines and scrape out the cells using a scraper.
4. Collect the scraped cells in PBS in an eppendorf tube, centrifuged and throw out the PBS supernatant.
5. Add 500 µl of magnesium containing protein lysis buffer (*See Annexure II, No. 3*) and subject the tubes were to rapid freeze thawing method by putting them in liquid nitrogen and thawing them on ice repeatedly thrice.
6. Centrifuging the samples at 12000 rpm for 15 min.
7. Take out the supernatant containing the protein in an eppendorf tube and stored at –20°C. Quantify the protein content by the BCA protein assay reagent kit.

B. Separation of Protein by SDS-PAGE

The gel percentage should be used depending on the size of the protein whose differential expression profile was to be studied. The gel concentration and protein size are inversely correlated. Adequate equal quantity of protein from samples to be compared are firstly denatured with Laemmli buffer 2X (*See Annexure II, No. 13*), and then loaded in the SDS-PAGE.

For details of this procedure follow *Chapter VI; Experiment 8*.

Also consult *Chapter I; j-Electrophoresis*.

C. Immunoblotting

1. Allow the SDS-PAGE to run at 60 V for 90 min or till the blue front comes out of the gel in a running buffer (*See Annexure II; No. 18*).
2. Once the run is complete, take out the gel carefully and subjected to wet transfer to a

nitrocellulose membrane (e.g. HYBOND) in a western blot transfer system for a standardized adequate time at 33 V depending on the size of the protein to be transferred. For performing this, arrange the gasket or cassette supplied with the Western blotter in a serial order: sponge → Whatmann filter paper → nitrocellulose/nylon membrane → poly-acrylamide gel → Whatmann filter paper. Fit the cassette in the respective slot in the Western blotter system in such a way that the membrane faces the anode and the gel faces the cathode end. Pre-fill the blotting tank with the transfer buffer.

3. After the transfer is complete, put for overnight blocking using adequate 5% BSA in PBST, on a shaking platform at 4°C.
4. Next day, wash the blot thrice (10 min each) in PBST.
5. The washing should be followed by addition of specific antibodies diluted in 1 ml blocking solution to the membrane and spread out properly.
6. On a plain clean glass surface, cover the membrane with a parafilm carefully such that no air bubble is trapped beneath, and leave undisturbed for overnight at 4°C or at room temperature for 1–2 h as per the recommendations of the datasheet provided by the antibody manufacturers.
7. Wash the blot repeatedly (thrice 10–15 min each) in PBST to wash out the unbounded antibodies.
8. Add the HRP labelled secondary antibody diluted in blocking buffer (*See Annexure II; No. 21*) on the membrane surface and keep for 1–2 h at room temperature, depending on the specificity of the primary antibody used for the protocol.
9. Wash the membranes thrice with PBST.
10. Wrap the washed membrane with a suranwrap and develop the membrane using the Western blotting developing kit (e.g. ECL plus, Amersham) following the kit manufacturer's protocol.

11. Capture the chemiluminescence based results by exposing X-ray films to the blots for specific time intervals (10 sec to 5 min).
12. Develop the X-ray film (in dark or low intensity red light) in developer till bands appear (1–2 min). Wash in running tap water and fix it using a fixer. Wash it under running tap water and air dry the X-ray blot.
13. Analyze the film for target protein differential expression in normal light.

Observation

The X-ray blot/film depicting the expression of a specific protein expression in different cases (e.g. healthy primary cell line v/s disease cell line) is analyzed manually; e.g. as shown in the figure below:



Viva Questions



- What does the X-ray films indicate by capturing it to the chemiluminescence based results?
- How does target protein of normal (or healthy primary cell line) cells differ from diseased cells?
- Also see Chapter VI; Experiment 8.



Experiment 5

Aim

Immunology: Bacterial antigen preparation—Preparation of antigens from bacterial culture routes of inoculation of antigens in rabbit/mice by different routes of immunization.

Key Terminology

- **Antigen** The smallest component that can evoke an immune response. It is also the general term for a substance that binds with a specific antibody.
- **Antibody** The molecules that are developed by the body's immune system to fight against the antigen.
- **Immunization** Administration of an antigen (or its parts) in small doses, so that the body acquainted to the antigen and thereby

Manually, we can notice the higher expression of target protein in disease cases compared to control cases. For absolute quantification, one needs to quantify using densitometry and suitable software analysis.

Results

The results from the immunoblot/Western blotting experiment shows that the target protein _____ is expressed more (_____ folds) in disease cases compared to controls and therefore can be predicted to be associated with the pathogenesis of the disease.

Precautions

1. As proteins are temperature sensitive, preferably the procedure till the transfer should be performed at 4°C.
2. Wear gloves throughout the procedure.
3. Don't expose the Chemiluminescence kit materials or the X-ray films to light directly.
4. Don't cross-contaminate or expose the antibodies to high temperatures.

allowing to detect, respond and memorize the antigen through the production of antibodies which are specific to the antigen, therefore pre-preparing the body to resist and fight against any attack in the future by the same specific antigens. This complete procedure is termed as immunization.

Basic Principle

The antigen can be administered into an animal body by a variety of routes. The main consideration while selecting a route is form and nature of the

antigen, and form and dose of antigen. Antigen may be of several types, viz. soluble and particulates and entire complex cells such as tumour cells and bacteria. Material to be injected should be kept sterile (soluble materials can be made sterile by passing through Millipore disc). Particulate antigens can be sterilized by using 0.5% phenol or Merthiolate (0.1%). Proteins can be usually be given in wide dose range (1 µg to 1 mg per animal) to raise antibodies against cell surface antigen (1 in 100 million cells per animal cell used).

Routes of Immunization There are different routes of administration of antigen, they are:

- (a) Intradermal
- (b) Subcutaneous
- (c) Intramuscular
- (d) Intraperitoneal
- (e) Intravenous

Materials

- Animals (rabbit/mice)
- Antigen
- Syringe (2 ml needle)
- Cotton
- Spirit

Methods

A. Intradermal Route This route is generally used for injecting viscous and slowly dispersing form antigens. It provides rapid access to the lymphatics

- (i) The area to be inoculated is cleaned with 70% alcohol
- (ii) The solution to be injected is filled into the syringe
- (iii) By holding a fold of the skin between the forefinger and the thumb the point of the needle is inserted into dermis parallel to skin surface and the solution is injected.
- (iv) The needle is then withdrawn.

B. Subcutaneous Route This route is suitable for emulsion precipitates and viscous material

- (i) The skin of the belly is cleaned with 70% alcohol.

- (ii) By holding a fold of skin between the thumb and index finger, the needle is inserted into the pocket of skin lying beneath the skin fold taking care not to pierce the peritoneum.
- (iii) The desired volume of solution is injected and the needle is withdrawn.

C. Intramuscular Route It is one of the most frequently adopted route of immunization and suitable for alum precipitates and absorbed antigens.

- (i) The needle is inserted from the rear at right angles to the skin surface, at a point half way along the femur.
- (ii) The inoculation is made, the needle withdrawn and the site is gently massaged.

D. Intraperitoneal Route It is more suitable for complex and particulate antigen (e.g. cell suspension and antigen which require processing)-

- (i) The skin is held as a subcutaneous injection and the needle inserted into the abdomen.
- (ii) The required volume is inoculated, the needle withdrawn and the site of needle entry pinched.

E. Intravenous Route This route requires extra care as antigen directly enters the blood circulation. It is good for particulate antigen. In case of rodents, tail vein is used for intravenous inoculation

- (i) the tail is held and needle inserted so that it follows directly along the line of the vein and comes to lie within the lumen
- (ii) the solution is injected and the needle withdrawn

B. Screening for Immunization

The first step of the screening process involves bleeding of the animal. Blood can be collected in one of the following ways

1. bleeding from the ear
2. retro orbital bleeding
3. cardiac puncture
4. from bronchial vein
5. from external jugular vein



Experiment 6

Aim Screening for the development or formation of the primary antibody against the inoculated (administered) antibody by **A.** 'Haemagglutination' and **B.** 'Immuno-diffusion' methods.

A. Haemagglutination Method

Key Terminology

- **Haemagglutination** Cross-linking of cells or large particles by antibody directed against surface antigen leads to agglutination.

Materials

- Microtitre plate
- Micropipette
- Disposable tips
- Diluting buffer (PBS, pH=7.2) (See Annexure II, No.16),
- Sample of animal RBC (1% animal RBC in PBS, pH 7.2)
- Antigen

Methods

1. The wells of the micropipette plate should be first filled with 100 μ l of PBS.
2. Add 100 μ l of antigen to the wells in serial dilution. Leave the last two rows of the plate as controls.
3. Prepare a 1% sample of the animal RBC (blood collected from inoculated animal) in PBS and add 100 μ l to each well.
4. Keep the plate undisturbed for 30 min at room temperature.
5. Inspect the plate visually. The wells with the serially diluted antigen are studied against the control wells.

Observation

All the wells are observed for the button formation at the bottom by comparing with the control wells. The wells with diluted antigen shows agglutination formed in decreasing order across the rows and button forming in increasing order.

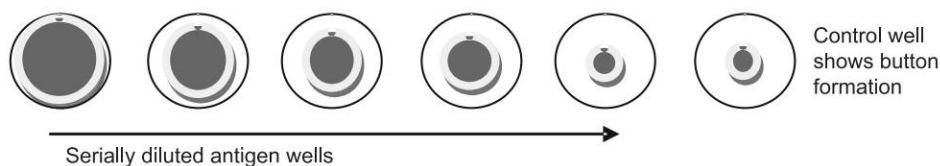


Fig. 8.6.1 The haemagglutination process showing decreasing degree of agglutination in a microtitre plate

Result

The increased button formation compared to the controls is indicative of the proper antigen-antibody reaction and establishes the development of primary antibody against the inoculated antigen in the immunized animal model.

B. Immuno-diffusion Method

Basic Principle

Agar gel diffusion is the simplest and direct means of demonstrating soluble antigen-antibody reaction in a laboratory. It may be classified as

single or double. In single agar gel diffusion, either antigen or antibody remains fixed or the other reactant is allowed to diffuse freely in the gel. In the double diffusion both the reactants are free to move towards each other and precipitate at the *zone of equivalence* which is seen as a white arc in the gel.

Materials

- Agarose gel: 1% in PBS (0.05 M, pH 7.2)
- Clean slide
- Puncturing needle
- Centrifuge

Methods

1. Serum preparation:
 - (a) After collecting, blood should be allowed to clot for 60 min at 37°C or overnight at 4°C.
 - (b) Spin at 10000 xg for 10 min. at 4°C to separate the serum.
 - (c) Serum can be stored at -20°C after adding Glycerol to 50%.
2. Clean a glass slide by wiping with acetone and carefully place on a flat horizontal surface.
3. Warm about 3 ml of agarose. With the help of a pipette carefully release the agarose (molten) at the centre of the slide. Allow the agarose to solidify for 15 min.
4. Puncture two wells in the agarose gel at about a distance of 1–1.5 cm from each other.
5. Fill one of the wells with the antigen solution, while in the other fill with the serum presumed to contain the antibody against the antigen.
6. Incubate the slides overnight at 37°C to allow diffusion by placing them in a petriplate lined with wet filter paper to avoid dehydration.
7. Observe the slides on the second day.

Observation

An arc of precipitate at the zone of equivalence between antigen-antibody should be seen in the

double immuno-diffusion slides.

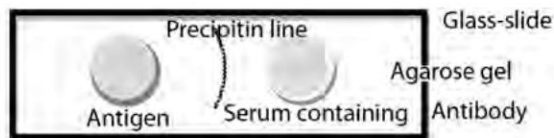


Fig. 8.6.2 Double immuno-diffusion slide showing precipitin line formation due to antigen-antibody reaction at the zone of equivalence

Result

The successful immuno-diffusion assay by virtue of the precipitin line formation is indicative of the proper antigen-antibody reaction and establishes the development of primary antibody against the inoculated antigen in the immunized animal model.

Precautions

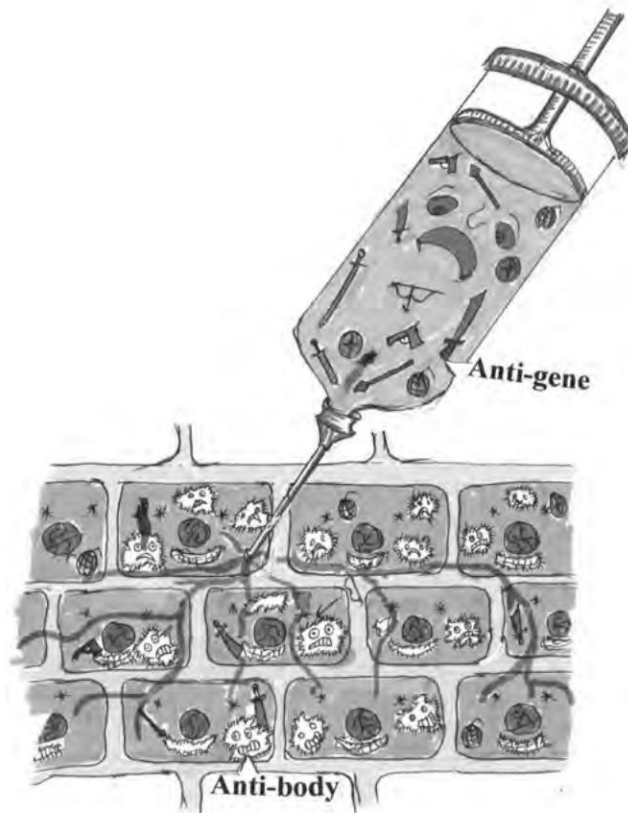
- Animals need to be carefully handled under supervision at the time of inoculation as well as bleeding. Take ethical committee permission if required.
- Dose estimation needs to be very proper for different animal models.
- For antibody generation screening, bleed the animal 7–10 days later after inoculation and test serum using the suitable assays for screening. If titre is not high enough, boost again two weeks after previous boost with the antigen.

Viva Questions

1. Define the basic principle of Haemagglutination.
2. What are the alternative protocols of studying antigen-antibody interaction?



Notes and Sketches

[illegible]

Annexures

ANNEXURE I

List of Equipments required for conducting Biotechnological practical

Sl.No	Name of the Equipment and Detail	Remarks
1.	Incubator (Temp. range 20° to 80°C) or Oven (Range 80° to 100°C) for drying and Sterilization	
2.	Vortex mixer	
3.	pH-meter (Digital)	
4.	Autoclave (Vertical and Portable) or Pressure cooker (Big)	
5.	Refrigerator (Cap. 400 Lit.) with Double door	
6.	Homogenizer	
7.	Eppendorf or Refrigerated Centrifuge (Table top type) Speed 15,000 rpm with 2 or 3 Rotar; Rotar size-2, 15 and 50 ml	
8.	Electrophoresis System—Complete set (Vertical and horizontal type) with Power Pack (Tank size—100 to 250 ml)	
9.	Micro Pipetman (Size—0.1 to 2.5, 2–20, 20–200, 100 to 1000 µl)	
10.	UV-vis-Spectrophotometer (Wavelength 200 to 700 nm)	
11.	UV-Trans-illuminator (with Safety Glasses and Mask) (With or without Photographic attachments)	
12.	All Glass-Double Water Distillation Plant (Cap. 3 Lit.)	
13.	Laminar-Flow Sterile Cabinet or Table Top (Horizontal type fitted with UV-lamp).	

14. Filter Sterilization Unit, Vacuum Pump (With 0.22 µ Diameter Filter for Ultra-filtration)
15. Single Pan Digital Balance (Sensitivity 0.1 mg)
16. Compound Microscope (With 5X, 10X and 15X and 100X Eyepieces)
17. Magnetic Stirrer with Hotplate
18. Incubator Shaker or Simple Shaker
19. Thermo Cycler (PCR)
20. ELISA Kits
21. Inverted Microscope

NOTE: Number and Quality or Make depending on Fund

ANNEXURE II

Common Buffers usually used in Biotechnological experiments

Buffer is an aqueous solution consisting of a mixture of a weak acid to its conjugate base. It has the property that the pH of the solution changes very little when a small amount of acid or base is added to it. Buffer solutions are used as a means of keeping pH at a nearly constant value in a wide variety of chemical application (See Chapter I; d-Buffer).

Buffer Compositions

I. CTAB Extraction buffer

STOCK	[FINAL]	10 ml
Distilled water		6.5 ml
1 M Tris-7.5	100 mM	1.0 ml
5 M NaCl	700 mM	1.4 ml
0.5 M EDTA-8.0	50 mM	1.0 ml

CTAB	2 %	0.2 g
14 M BME	140 mM	0.1 ml

Adjust pH to 5.0 with 0.1N.HCl.

2. Tris EDTA (TE) buffer

10 mM Tris (pH 8.0)
1 mM EDTA (pH 8.0)

3. Lysis buffer

NH₄Cl -155 mM
KHCO₃ -10 mM
Na₂EDTA-0.1mM
Fill to 1000 ml with distilled water
Adjust to pH 7.4 with 1 M HCl or NaOH for each use

4. SE-Buffer

NaCl - 75 mM
Na₂EDTA - 25 mM
Fill to 1000 ml with distilled water
Adjust to pH 8.0 with 1 M NaOH for each use

5. 10xTBE (2l)

218.0 g Tris base
110.0 g Boric acid
9.3 g EDTA
Double distilled water—make the volume up to 2l
pH to about 8.3 using NaOH and make up to 2l.

6. Grinding solution

0.33 M Sorbitol
10 mM Sodium pyrophosphate (Na₄P₂O₇)
4 mM MgCl₂
2 mM Ascorbic Acid
Adjust pH to 6.5 with HCl

7. HEPES or Suspension solution buffer

0.33 M Sorbitol
2 mM EDTA
1 mM MgCl₂
50 mM HEPES
Adjust pH to 7.6 with NaOH

8. Magnesium containing protein lysis buffer (MLB buffer)

25 mM HEPES, pH7.5
150 mM NaCl
1% NP₄₀

0.25% Na deoxycholate
10% glycerol
25 mM NaF
10 mM MgCl₂
1 mM EDTA

The above protein lysis buffer should be prepared and stored at 4 °C, 10 µg/ml Leupeptin, 10 µg/ml Aprotinin and 5 µg/ml PMSF (all from SIGMA) as protease inhibitors are added just before use.

9. 30% Acrylamide solution stock

Acrylamide 29.2 g
Bis-acrylamide 0.8 g
ddH₂O 100 ml
Keep at 4 °C.

10. Na-Citrate buffer

100 ml of 0.15 M NaCl and 100 ml of 0.015 M of Na-citrate solution was taken and mixed thoroughly and pH adjusted to 7.0.

11. 2 X HEPES-buffered saline

1. Dissolve 1.6 g NaCl, 0.074 g KCl, 0.027 g Na₂HPO₄·2H₂O, 0.2 g dextrose, and 1 g HEPES.
2. Add 90 ml distilled water.
3. Adjust to pH 7.05 with 0.5 M NaOH.
4. Make up volume to 100 ml with distilled water.
5. Sterilize the solution by filtration through a 0.22 micron filter.
6. Store in 5 ml Aliquot at -20 °C.

12. 10X Assay buffer (buffer for restriction digestion)

50 mM
Tris-HCl (pH 7.9 at 37°C)
250 mM NaCl
50 mM MgCl₂
5 mM DTT
0.5 mg/ml acetylated BSA

13. Laemmli buffer

4% SDS
10% Beta-mercaptoethanol
20% glycerol
0.125 M Tris HCl
Adjust pH to 6.8

14. 10X PCR Amplification buffer

PCR buffer is necessary to create optimal conditions for activity of Taq DNA polymerase. Buffers often contain Tris-HCl, KCl, and sometimes MgCl_2 . PCR buffers are often available in 10X concentration and are sometimes Taq formulation-specific. The 10X PCR buffer contains 500 mM KCl; 100 mM Tris-HCl (pH 8.3); 15 mM MgCl_2 .

15. 2X Ligase Buffer

60 mM
Tris-HCl (pH 7.8 at 25°C)
20 mM MgCl_2
20 mM DTT
2 mM ATP

Store in single-use aliquots at -20°C.

16. (a) Phosphate Buffered Saline (PBS) 10X PBS (0.1 M MQ PBS, pH 7.2)

Na_2HPO_4 (anhydrous)—10.9 g
 NaH_2PO_4 (anhydrous)—3.2 g
NaCl—90.0 g
Distilled water—1000 ml

Mix to dissolve and adjust pH to 7.2. Store this solution at Room temperature. Dilute 1:10 with Distilled water before use and adjust pH if necessary.

or (b) Dissolved the following in 800 ml MQ H_2O + 8.0 g NaCl + 0.2 KCl + 1.44 g

Na_2HPO_4 + 0.24 g KH_2PO_4 .

Adjust the pH to 7.4. Add MQ H_2O to 1 l. Sterilize to autoclave. Store at room temperature.

17. Transfer buffer (1.5 l)

Tris 4.5 g
Glycine 21.6 g
Adjusted the volume up to 1.2 l by adding MQ water
Methanol 300 ml

18. Running buffer (1 L)

Tris 3.0 g
Glycine 14.4 g
20% SDS 5 ml
Adjusted up to 1L by adding MQ water

19. 5X RT buffer

Commercially supplied free of cost along with the reverse transcriptase enzyme.

20. Digestion buffer

Commercially supplied free of cost along with the restriction enzyme.

21. Blocking buffer

5% bovine serum albumin in PBST.

Calcium- and Magnesium-Free Phosphate-Buffered Saline (CMF-PBS).

22. SE buffer

NaCl 75 mM
Sodium EDTA 25 mM
(pH-8)

23. Tris Borate EDTA (TBE)

(See chapter 1, page 20)

NOTE: These buffers are provided free of cost along with the respective enzymes when procured from any recognized company.

Composition of**1. 6X alcohol-orcinol:**

Weigh 15 g orcinol reagent and dissolve in 25 ml of 95% alcohol and store in dark.

ANNEXURE III**Stains and Nutrient Media composition for Microbiological experiments****Stains usually used in Microbiological studies****1. Gram's Iodine**

Dissolve 20 g of potassium iodide in 250 ml distilled water and then add 10 g of Iodine. Make up the volume to 1 l with distilled water.

For 100 ml:

Use Lugol's iodine, 3.0 ml in 100 ml of Distilled water or add 1.0 ml Iodine and 2.0 g to 100 ml of Distilled water.

2. Saffranin stain Prepare 0.5 per cent Saffranin in distilled water.

3. Crystal Violet stain Dissolve 10 g Crystal Violet in 100 ml absolute alcohol. Make up the volume to 1 Litre with distilled water.

Some Common Nutrient Media Preparation used for Bacteria Culture

1. Trypticase Soya Broth/Agar culture (TSB/TBA):

- | | |
|----------------------------|----------|
| (a) Peptone from casein | 15.0 g/l |
| (b) Peptone from soya meal | 5.0 g/l |
| (c) NaCl | 5.0 g/l |
| (d) Agar-agar | 15.0 g/l |

Preparation (a) Suspend 40.0 g/l (For Agar culture) or 30.0 g/l (For Broth culture). Autoclave for 15 min at 121°C. Maintain pH to 7.2 ± 0.2 at 25°C. Incubate for 24 h at 25°C aerobically.

2. Starch Agar Plate (For 20 plates):

- Add 2.0 g of soluble Starch to 400 ml of distilled water.
- Add 9.2 g of Nutrient Agar and Autoclave for 15 min at 121°C and dispense in plates

3. Skim Milk Agar Plate *

- Peptone from Erosin 5.0 g/l (Tryptone)
- Yeast extract 2.5 g/l
- Skim milk powder 1.0 g/l
(No inhibitors, antibiotic free)
- Glucose 1.0 g/l (Dextrose)
- Agar-agar 10.5 g/l

Preparation (a) Suspend 20.0 g/l in cold water and allow to stand for 15 min. Transfer flux to a cold water bath and heat gently, with frequent shaking until completely dissolved.

- Maintain pH 7.0 ± 0.2 at 25°C and then autoclave for 15 min at 121°C.

*NOTE: (a) Addition of skim milk to a culture medium with a superior nutrient base optimally adopts it to the neutral conditions experienced by micro-organism which grow in milk. More colonies grow and a wider range of bacteria can be cultured than in other culture media intended for the same purpose.

- The plates are clear to opalescent and yellowish. The reconstituted culture medium is more or less opalescent. According to Deutsches Institut für Normung (DIN) it can be stored up to 3 months in refrigerator, the temperature should not exceed 5°C.

4. Potato Dextrose Agar or Saboraud Dextrose Agar (SDA)

- | | |
|---------------------|---------------------------------------|
| (a) Potato infusion | –4.0 g/l (Infusion from 200 g potato) |
| (b) D(+) glucose | –20.0 g/l |
| (c) Agar agar | – 15.0 g/l |

Preparation Suspend 39 g/l autoclaved for 15 min at 121°C; pH 5.6 ± 0.2 at 25°C. If the pH has to be adjusted to 3.5, add approximately 14 ml of a sterile 10% Tartaric acid solution per litre at a temperature of 45–50°C. The plates are clear and yellowish-brown in colour. Do not reliquefy.

Mode of Action Used for the cultivation, isolation and enumeration of yeast and moulds from food stuffs and other materials. Carbohydrate and potato infusion promote the growth of yeast and moulds, while the low pH value partially inhibits the growth of the accompanying bacterial flora. If the medium is to be used for fungal counts, the pH should be adjusted to approximately 3.5 ± 0.2 . Fungi grow on this medium to develop typical morphology.

Procedure for Evaluation Inoculate by the pour-plate method or by spreading the sample on the surface of the culture medium. Incubate up to 5 days at 28°C, aerobically. Experimental procedure depends on the purpose for which medium is used.

Quality Control for Following Organism

Penicillium commune, *Geotrichum candidum*, *Trichophyton ajelloi*

Spiral Plating Method *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus niger*.

5. Phenol Red Lactose / Maltose Broth

- | | |
|----------------------|---------------------|
| (a) Proteose peptone | – 10.0 g/l |
| (b) Beef extract | – 1.0 g/l |
| (c) Sodium chloride | – 5.0 g/l |
| (d) Phenol red | – 0.018 g/l |
| (e) Lactose/maltose | – 5.0 g/l (Any one) |

Final pH 7.4 ± 0.2 at 25 °C.

Preparation Suspend 21 g pf Phenol Red Lactose/ Maltose (any one) broth in 1000 ml of distilled water. Heat to dissolve the medium completely. Dispense into tube containing inverted Durham's tube and sterilize by

autoclaving at 15 lb pressure for 15 min at 121°C. Final preparation of the broth should be Red coloured, clear solution without any precipitation. Media is meant for laboratory use only.

Test organism

ORGANISM	PHENOL RED LACTOSE BROTH			PHENOL RED MALTOSE BROTH		
	GROWTH	ACID	GAS	GROWTH	ACID	GAS
<i>Enterobacter aerogenes</i>	Luxuriant	+	+	Luxuriant	+	+
<i>E. coli</i>	Luxuriant	+	+	Luxuriant	+	+
<i>Klebsiella pneumoniae</i>	Luxuriant	+	+	Luxuriant	+	+
<i>Proteus vulgaris</i>	Luxuriant	+	+	Luxuriant	–	–
<i>Salmonella typhimurium</i>	Luxuriant	+	+	Luxuriant	–	–
<i>Salmonella typhi</i>	Luxuriant	+	–	Luxuriant	–	–
<i>Serratia marsecense</i>	Luxuriant	+	–	Luxuriant	–	–
<i>Shigella flexneri</i>	Luxuriant	+	–	Luxuriant	–	–

6. Corn Meal Agar

- (a) Corn meal, infusion from solids –50 g
(b) Agar –15 g

Infusion from corn meal is the source of carbon, nitrogen and vitamins required for organism growth in corn meal agar.

Note: Formula may be adjusted and/or supplemented as required to meet performance specification. Meant for laboratory use only. Some *Candida* spp. May take up to 14 days to produce chlamydo spores.

Preparation Suspend 17 g of the medium in one litre of purified water. Heat with frequent agitation and boil for one minute to completely dissolve the medium. Autoclave at 121°C for 15 min. Powder is homogeneous, free flowing granules and beige to yellow in colour. Prepared medium is to trace moderately hazy and grey to yellow-white in colour.

Culture Response It is used for the cultivation of fungi and the demonstration of chlamydo spore production. Culture on corn meal agar should be examined for chlamydo spores production after 2 to 7 days of incubation at 25 ± 2°C.

MICROORGANISM	RESPONSE	RESULTS
1. <i>Aspergillus niger</i>	Growth	N/A
2. <i>Candida albicans</i>	Growth	+ve Chlamydo spore production
3. <i>Saccharomyces cerevisiae</i>	Growth	–ve Chlamydo spore production

7. Mycosel Agar

- (a) Papaic digest of soybean meal – 10.0 g/l
(b) Dextrose – 10.0 g/l
(c) Agar – 15.5 g/l
(d) Cycloheximide – 0.4 g/l
(e) Chloramphenicol – 0.05 g/l

Is a highly selective medium containing cycloheximide and chloramphenicol. It is recommended for the isolation of pathogenic fungi from material having a large flora of other fungi and bacteria. The plates are deep filled to reduce the effect of drying during prolonged incubation.

Preparation from Dehydrated Powder Suspend 36 g of the powder in one litre of purified water. Mix thoroughly. Heat with frequent agitation just until the medium boils, to completely dissolve the powder. Autoclave at 118°C for 15 min. Avoid overheating. Test samples of the finished product for performance using stable, typical control culture. Colour of the finished medium will be light to yellow or tan, clear to moderately hazy.

Culture Response The nutritive properties of mycosel agar are supplied by the peptone prepared from soybean meal. Dextrose is an energy source for the metabolism of fungi. Cycloheximide inhibits most saprophytic moulds. Chloramphenicol is a broad spectrum antibiotic which inhibits a wide range of gram positive and gram negative bacteria.

Prepare the medium as per direction, inoculate with fresh cultures and incubate at 25 ± 2°C for 7 days. For isolation of causing systemic mycoses, two sets of media should be incubated, with one set incubated at 25–30°C and a duplicate set at 35 ± 2°C. All cultures should be examined at least weekly for fungal growth and should be held for 4–6 weeks before reported as negative.

The following organisms show good growth

- (i) *Phialophora verrucosa*
(ii) *Microsporum audouinii*

- (iii) *Candida albicans*
- (iv) *Blastomyces dermatitidis*

Partial to complete inhibition

- (i) *Aspergillus niger*
- (ii) *E. coli*
- (iii) *Aureobasidium pullulans*
- (iv) *Penicillium rowuefortii*
- (v) *Staphylococcus aureus*
- (vi) *Streptomyces rimosus*

8. Motility Test Medium

- (a) Beef extract – 3.0 g/l
- (b) Pancreatic digest of casein – 10.0 g/l
- (c) Sodium chloride – 5.0 g/l
- (d) Agar – 4.0 g/l

Preparation Suspend 22 g of the powder in one litre of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 min to completely dissolve the powder. Dispense and autoclave at 121°C for 15 min. If desired, 5 ml of sterile 1% TTC solution may be added aseptically after autoclaving. Test samples of the finished product for performance using stable, typical control culture. Finished medium is pale to light, yellow to tan, clear to slightly hazy in colour.

Culture Response Motility test medium is used for the direction of motility of gram negative enteric bacilli. Prepare the medium as per direction. Stab the inoculate with fresh cultures and incubate at $35 \pm 2^\circ\text{C}$ for 2 days.

Bacterial motility can be observed directly from examination of the tubes following incubation. Inoculate tubes with a pure culture by stabbing the centre of the column of medium to greater than half the depth. Incubate tubes for 24–48 h at $35 \pm 2^\circ\text{C}$ in an aerobic atmosphere. After incubation, observe the tubes for growth in relation to the stab line. Non-motile organisms grow only along the line of inoculation, while motile organisms spread out from the line of inoculation and may even grow throughout the medium. Negative tubes can be reinoculated at $25 \pm 2^\circ\text{C}$ for an additional 5 days, if desired.

Limitation Many organisms fail to grow deep in semisolid media; inoculating the pour plates may be advantageous.

ORGANISM	RECOVERY	MOTILITY
<i>Enterobacter aerogenes</i>	Good	+
<i>E. coli</i>	Good	+
<i>Kebsiella pneumoniae</i>	Good	–
<i>Shigella flexneri</i>	Good	–
<i>Proteus vulgaris</i>	Good	+

9. * Nutrient Agar Plates

- (a) Bacto peptone – 1.0 g/l
- (b) KH_2PO_4 – 2.0 g/l
- (c) NaCl – 5.0 g/l
- (d) K_2HPO_4 – 5.0 g/l
- (e) Bacto Agar – 10.0 g/l

Preparation Dissolve 23 g in one litre distilled water. Adjust pH to 7.0. Sterilize by autoclaving at 121°C for 15 min. Medium is used in Molecular biology for cultivation technology of *E. coli* strains for the preparation of phage and plasmid DNA.

Smaller volumes than a liter can be made by scaling down the quantity of each ingredient by the same ratio as the volumes. To vary the nutrient levels in this experiment, vary the Bacto Peptone levels in the solution. For streak plates, you can use a concentration of 1.0g/l Bacteriological Peptone. There are many other kinds of Nutrient agar that can be used to obtain individual colonies. Since bacteria grow differently on different kinds of Nutrient agar, you may find that one works better than another for you. Generally used for isolation of bacteria and Actinomycetes.

*One standard Nutrient Agar is Luria-Bertani (LB) media given below:

10. * Luria Bertani (LB) culture of *E. coli*

- (a) Tryptone – 10.0 g/l
- (b) Yeast extract – 5.0 g/l
- (c) Sodium chloride – 5.0 g/l
- (d) Agar agar – 10.0 g/l (Not needed for LB broth)

Note: In Broth culture supplement the LB medium with Kanamycin, Zeocin and Ampicillin. These products make the medium more selective to recombinant *E. coli*.

Preparation Adjust the pH to 7.2 at 37°C. Store prepared media below 8°C, protect from direct light. Store dehydrated powder, in a dry place, in tightly sealed container at 2° to 25°C.

Dissolve 30 g in a litre of distilled water and adjust the pH to 7.2. Sterilize by autoclaving at 121°C for 15 min.

Mode of Action For the culture of *E. coli* K12 strain for the preparation of phage and plasmid DNA. Medium favours lysogeny. It is also a general medium for *E. coli* in fermentative molecular genetic studies and may be used for routine cultivation of not particularly fastidious microorganisms.

11. *Luria Bertani Top Agar

- | | |
|---------------------|------------|
| (a) Tryptone | – 10.0 g/l |
| (b) Yeast extract | – 5.0 g/l |
| (c) Sodium chloride | – 10.0 g/l |
| (d) Agar agar | – 7.0 g/l |

(* Same as other LB culture)

Preparation Dissolve 32 g in one litre distilled water. Adjust pH to 7.0. Sterilize by autoclaving at 121°C for 15 min. Cool to 48°–55°C, and then add the bacterial suspension. Immediately mix and pour the suspension on LB-Agar plates.

Medium is used in molecular biology for cultivation technology of *E. coli* strains for the preparation of phage and plasmid DNA.

12. Eosin Methylene Blue Agar (EMB Agar)

- | | |
|----------------------------------|------------|
| (a) Pancreatic Digest of Gelatin | – 10.0 g/l |
| (b) Eosin Y | – 0.4 g/l |
| (c) Lactose | – 10.0 g/l |
| (d) Methylene blue | – 0.06 g/l |
| (e) Dipotassium phosphate | – 2.0 g/l |
| (f) Agar | – 10.0 g/l |

EMB Agar is used in the identification of *E. coli* and other gram-negative enterobacteria. It is both a selective and differential agar. EMB agar is inhibitory to gram-positive bacteria, and is indicative of lactose fermentation. It is selective because it contains Bile salts and eosin and Methylene blue dyes, which inhibit gram-positive bacteria from growing. It is also differential, in the Lactose-fermenting bacteria, such as *E. coli* will produce dark colonies with a green metallic sheen, while bacteria that cannot ferment lactose produce colourless colonies.

13. Brilliant Green Phenol-Red Lactose, Sucrose Agar Modified:

- | | |
|-----------------------|----------|
| (a) Peptone from meat | 10.0 g/l |
|-----------------------|----------|

- | | |
|---------------------------------|------------|
| (b) Meat Extract | 5.0 g/l |
| (c) Yeast extract | 3.0 g/l |
| (d) Disodium Hydrogen Phosphate | 1.0 g/l |
| (e) Sodium dihydrogen Phosphate | 0.6 g/l |
| (f) Sucrose | 10.0 g/l |
| (g) Phenol Red | 0.09 g/l |
| (h) Brilliant Green | 0.0047 g/l |
| (i) Agar-agar | 12.0 g/l |

Preparation Suspend 51.5 g in 1 l purified water feed gently with frequent agitation and bring to boil to dissolve completely afterwards pour in plates at ~50°C. **Do not autoclave.** Maintain pH to 6.9 ± 0.2 at 25°C. Incubate for 24 h at 35°C aerobically.

Appearance of Colonies (a) Red, surrounded by a light red zone.

Test strains *Salmonella typhimurium*; *Proteus vulgaris*; *Pseudomonas* and others. (Lactose and sucrose –ve).

(b) Yellow, surrounded by yellow zone.

Test strains *Escherichia coli*; *Enterobacteria*; *Klebsiella*. Lactose and sucrose +ve.

14. KOVAC's reagent for Indoles

(a) N-butanol, (b) HCl, (c) 4-(dimethyl-amino) benzaldehyde

Preparation

- Add 0.2–0.5 ml Kovac's reagent to 5 ml of 24–48 h old culture, e.g. incubated in Tryptone H₂O.
- The inoculating more cell material in a smaller volume, e.g. (0.5 ml) shaking slightly to help extraction.

Quality control Cultural response after 18–24 h at 35°C in tryptone H₂O. If a cherry red colouration develops in less than a minute, it will be considered a +ve rn and means that the microorganisms possess tryptophanase activity. A –ve rn shows no colour change.

Kovac's reagent recipe:

- Add 25 ml of concentration HCl to 75 ml of allyl alcohol.
- Dissolve 5 g of p-dimethyl amino-benzaldehyde in the solution from Step (i).
- Aliquot to single use vials.
- Store at 4°C in closed vials.

Note: For determination of the ability of microorganisms, primarily *Enterobacteriaceae*, to split indol (benzopyrrol), from the Tryptophane molecule by Tryptophanase. Tryptophanase present in e.g. *E. coli* cleaves tryptophane to Indol and α -amino propionic acid. P-amino-benzaldehyde present in the reagent builds with Indol a cherry red complex, soluble in alcohol, ether and chloroform. For the test it is recommended to use media without glucose and a peptone with high tryptophane content.

15. Sulphide, Indole, Motility Medium

(a) Peptone from casein	– 20.0 g/l
(b) Peptone from meat	– 6.6 g/l
(c) Sodium thiosulphate	– 0.2 g/l
(d) Ammonium iron (II) citrate	– 0.2 g/l
(e) Agar agar	– 3.0 g/l

Preparation Suspend 30 g/l, dispense into tubes to give a depth of about 4 cm. Maintain pH: 7.3 ± 0.2 at 25°C and autoclave (15 min at 121°C). Allow to solidify in a vertical position. The prepared medium is clear and Yellowish-brown.

Note: Test culture medium used to detect sulphide formation, indole production and motility for the identification of *Enterobacteriaceae*.

16. Dermatophytes Test Medium (DTM)

(a) Peptone from soymeal	– 10.0 g/l
(b) D(+) Glucose	– 10.0 g/l
(c) Cycloheximide	– 0.5 g/l
(d) Gentamicin sulphate	– 0.1 g/l
(e) chlorotetracycline	– 0.1 g/l
(f) Phenol red	– 0.2 g/l
(g) Agar agar	– 17.0 g/l

Preparation Suspend 38 g in one litre of purified water. Autoclave under mild condition (10 min at 121°C) pour plate or prepare slant tubes. pH 5.5 ± 0.2 at 25°C . The plates are clear and yellow-orange.

Mode of action Culture medium proposed for the isolation and rapid differentiation of dermatophytes from specimens including those infected with other microorganisms. Selective DTM is superior to that of other media used for cultivating fungi. This medium offers the advantage that the dermatophytes grow rapidly and produce an unmistakable colour change.

This culture medium contains the pH indicator phenol red and the selective inhibitors cycloheximide.

Gentamicin and Chlorotetracycline, which partly suppress the growth of bacteria, yeast and moulds. When grown on DTM, most dermatophytes produce basic metabolites, which bring about an alkalization of the acid culture medium, causing the Phenol red to change its colour from yellow to red. This colour change may, however, occasionally be caused by other organisms too. Many moulds produce acidic metabolites, which do not change the colour of the culture medium. It is thus possible to differentiate rapidly between dermatophytes and other fungi with a high degree of accuracy (Approx. 97%).

17. MaConky's Broth Single and Double Strength for *E. coli*

(a) Peptone	– 17.0 g/l
(b) Protease peptone	– 3.0 g/l
(c) Lactose	– 10.0 g/l
(d) Bile Salt No. 3	– 1.5 g/l
(e) Sodium chloride	– 5.0 g/l
(f) Neutral Red	– 0.03 g/l
(g) Crystal violet	– 0.001 g/l
(h) Agar	– 13.5 g/l

Preparation Dissolve the peptone, sodium chloride and bile salt in water by heating. Adjust the pH to 8.0 and boil for 20 min. Cool, filter and adjust pH to 7.4. Add the lactose and indicator solution, tube in 5 ml portion to tabs containing inverted Durham's tubes. Sterilize by autoclaving at 115°C for 15 min.

18. YEP Medium (With and without antibiotics)

(a) Yeast extract	– 10 g/l
(b) Peptone	– 10 g/l
(c) NaCl	– 5 g/l

Preparation Adjust pH to 7.0 with NaOH. For solid medium, add agar 23 g/l.

Dissolve all the constituents of YEP medium in water and adjust pH to 7.0. Aliquot 50 ml each of medium in conical flasks and autoclave at 23 psi pressure, 121°C for 20 min. Cool the flasks to 50°C and add antibiotics. Prepare YEP medium in petri-plates and also maintain liquid broth without agar in flasks.

(a) YEP broth/agar	– 100 ml
Kanamycin sulphate @	– 0/5 ml of
50 $\mu\text{g/ml}$	– 10 mg/l stock
(b) Streptomycin sulphate @	– 1.0 ml of

N.B.: All stock solutions and dilutions should be prepared in double distilled water

Composition of Nutrient Media Relevant to Present Study (Quantity in mg/l)

	<i>MS</i>	<i>B₅</i>	<i>SH</i>	<i>NITSCH's</i>	<i>N₆</i>	<i>AE*</i>	<i>WPM</i>
Macro-nutrient							
NH ₄ NO ₃	1650	-	-	720	-	1200	400
NH ₄ H ₂ PO ₄	-	-	300	-	-	-	-
(NH ₄) ₂ SO ₄	-	134	-	-	463	-	-
KNO ₃	1900	2500	2500	950	2830	1900	-
CaCl ₂ .2H ₂ O	440	150	200	-	166	180	96
MgSO ₄ .7H ₂ O	370	250	400	185	185	370	370
KH ₂ PO ₄	170	-	-	68	400	340	170
NaH ₂ PO ₄ .H ₂ O	-	150	-	-	-	-	-
Micro-nutrient							
KI	0.83	0.75	1.0	-	0.8	0.75	-
H ₃ BO ₃	6.20	3.0	5.0	-	1.6	0.63	6.2
MnSO ₄ .4H ₂ O	22.30	-	-	25.0	-	2.20	-
MnSO ₄ .H ₂ O	-	10.0	10.0	-	3.3	-	22.3
ZnSO ₄ .7H ₂ O	8.60	2.0	1.0	10.0	1.5	4.0	8.6
Na ₂ MoO ₄ .2H ₂ O	0.25	0.25	0.1	0.25	-	0.025	0.25
CuSO ₄ .5H ₂ O	0.025	0.025	0.2	0.025	-	0.002	0.25
CoCl ₂ .6H ₂ O	0.025	0.025	0.1	0.025	-	0.002	-
Na ₂ EDTA.2H ₂ O	37.30	-	20.0	37.30	37.30	19.0	37.30
FeSO ₄ .7H ₂ O	27.80	-	15.0	27.80	27.80	14.0	27.8
EDTA Na-ferric salt	-	43.0	-	-	-	-	-
Organic							
Inositol	100	100	1000	100	-	100	100
Pyridoxine-HCl	0.50	1.0	0.50	0.50	0.50	1.00	0.50
Nicotinic acid	0.50	1.0	5.0	5.0	0.50	2.00	0.50
Thiamine-HCl	0.10	10.0	5.00	0.50	1.00	5.00	1.00
Glycine	2.00	-	-	2.00	-	2.00	2.00
Folic acid	-	-	-	0.50	-	-	-
Biotin	-	-	-	0.05	-	-	-
pH	5.8	5.5	5.8	5.8	5.8	5.8	5.8

MS (Murashighe and Skoog,1962); B₅ (Gamborg *et al.*, 1968); SH (Schenk and Hadebrant, 1972); Nitsch's (Nitsch and Nitsch, 1969); AE (von Arnold and Erickson,1981); N₆ (Chu *et al.*, 1968); WPM (McCrown and Lyod, 1981).

*Added (in mg/l) D-glucose 180, D-xylose 150, L-arabinose 150, L-glutamine 0.4, L-Alanine 0.05, L-cysteine 0.02, L-arginine 0.01, L-leucine 0.01, L-phenyalanine 0.01 and 34.2 g/l sucrose.

Knudson's C (1946) Basal Media Composition for Orchid seed Germination**Major elements**

- (a) Calcium nitrate $\{Ca(NO_3)_2 \cdot 4H_2O\}$ –1,000.00 mg/l
 (b) Potassium hydrogen phosphate (KH_2PO_4) –250.00 mg/l
 (c) Ammonium sulphate $\{(NH_4)_2SO_4\}$ –500.00 mg/l
 (d) Magnesium sulphate $(MgSO_4 \cdot 7H_2O)$ –250.00 mg/l

Minor elements

- (e) Manganese sulphate $(MnSO_4 \cdot 4H_2O)$ – 7.50 mg/l

In addition add

- Sucrose (2% of the total solution) –20.00 mg/l
 Bacto agar agar (1% of the total solution) –10.00 mg/l
 Adjust pH to 5.1 ± 0.1 with 0.1N.HCl and 0.1N. NaOH

Autoclave at $121^\circ C$ or 15 lbs pressure for 15 min.

Note: May be supplemented with 100–150 g banana or 100–200 ml coconut water, and 2g of vegetable charcoal per litre.

ANNEXURE V

Answers to the Problems given in Chapter II: Genetics and Chapter VI: Cell and Molecular Biology

Experiment 2

Key for human karyotype from Photographs Plate-I and Plate-II.

CHRO- MOSOME NO.	PLATE-I	PLATE-II	CHRO- MOSOME NO.	PLATE-I	PLATE-II
1	1 + 23	1 + 4	13	13 + 35	25 + 28
2	2 + 24	2 + 5	14	14 + 36	26 + 29
3	3 + 25	3 + 6	15	15 + 37	27 + 30
4	4 + 26	7 + 9	16	16 + 38	31 + 34
5	5 + 27	8 + 10	17	17 + 39	32 + 35
6	6 + 28	11 + 18	18	18 + 40	33 + 36
7	7 + 29	12 + 19	19	19 + 41	37 + 39
8	8 + 30	13 + 20	20	20 + 42	38 + 40
9	9 + 31	14 + 21	21	21 + 43	41 + 43
10	10 + 32	15 + 22	22	22 + 44	42 + 44
11	11 + 33	16 + 23	X	45	45
12	12 + 34	17 + 24	X or Y	46	46

Experiment 3

- (a) 3 Tall : 1 Short
(b) All Tall.
- (a) Yes, if the parents were heterozygous
(b) No, because neither parent has a brown-eye gene.
- 1 Yellow : 1 Green.
- (a) Dumpy-wing gene is recessive
(b) Both parents were heterozygous.
- (a) 150 (b) 300
- (a) F_1 individuals were Bb (Suppose Black B allele and White b) because segregating in 3:1 ratio (approx.)
(b) Black (B) allele is dominant to its recessive White (b)
(c) Test cross the F_1 (Bb) with (bb) white genotype individual.
- Wild-body colour (b^+b) crossed to black-body colour (bb) segregated in 1 wild-body colour : 1 black-body colour (Approx.)

Wild-body colour (b^+b) $\times$ Black-body colour (bb)

↓

Gametes: b^+b All b

Offspring: $1b^+b : 1bb$

Wild-body : Black-body

- (a) $\frac{1}{4}$,
(b) $\frac{1}{4}$,
(c) (3 Coloured + 1 Albino) (3 Straight + 1 Bent)
= 9 Coloured Straight : 3 Coloured bent: 3 Albino straight : 1 Albino bent.
- (a) 40 (b) 120 (c) 90 (d) 30
- 1 Normal size, Normal leaves : 1 Normal Crinkly : 1 Pigmy Normal : 1 Pigmy Crinkly.
- 135—Wild-body colour, Normal wing
45—Wild-body colour, Vestigial wing
45—Ebony-body colour, Normal wing
15—Ebony-body colour, Vestigial wing
- (a) F_1 progeny: Genotypic ratio = 1 AACC : 1 AACc : 1 AaCC : 1 AaCc.
Phenotypic ratio = All flowers in leaf axil and red flower colour.
(b) Test cross the F_1 plants with aacc plants will produce
AACC $\times$ aacc = All flowers in leaf axil, Red flowers
AACc $\times$ aacc = Segregation for Red flower colour
AaCC $\times$ aacc = Segregation for flowers in leaf axil
AaCc $\times$ aacc = Segregation for both the characters

13. (a) F_1 Genotypic ratio = 1 LI BB : 1 lI BB : 1 lI Bb : 1 llbb : 2 LI Bb : 2 lI Bb
 F_1 Phenotypic ratio = 3 Short and Black hair
 3 Long and Black hair
 1 Short and Brown hair
 1 Long and Brown hair
 (b) Genotype of one Parent—Short and Black hair (LI BB)
 Second parent—Long and Brown hair (llbb)
14. (a) aBC (b) ABC Abc (c) ABcD Abcd (d) ABcDe
 aBc Abc aBc ABcd aBcd ABcde
 abC AbC abC AbcD abcD AbcDe
 abc aBC abC aBcd abcd Abcde
15. (a) Round is dominant over wrinkled seeds,
 Yellow is dominant over green seed colour.
 (b) Cross the round and yellow colour seed plant with Wrinkled, green seed colour (Test-cross).
16. (a) 9/16 proportion of the plant will be round, yellow, starchy;
 (b) 4/16 or 1/4 proportion of the plant will be heterozygous for round, yellow, starchy;
 (c) 1/16 proportion of the plant will be homozygous for all three alleles.
17. (a) Parents: $+c+f \times cff$
 Gametes: $++ +f c+ cf$ All cf
 F_1 : $+c+f +cff cc+f ccff$
 (b) Parents: $+++f \times +cff$
 Gametes: $++ +f +f cf$
 F_1 : $+++f +c+f ++ff +cff$
 (c) $+c+f \times +cff$
 Gametes: $++, +f, c+, cf$
- | | | | | |
|----|------|------|------|------|
| | ++ | +f | c+ | cf |
| +f | +++f | ++ff | +c+f | +cff |
| cf | +c+f | +cff | Cc+f | ccff |
- Experiment 5**
18. Absence of dominance; Pink flower is heterozygous. Thus, Pink $\times$ Pink gives a ratio of: 1 Red : 2 Pink : 1 White.
19. F_1 : Long ($S^L S^L$) $\times$ Oval ($S^L S^R$) = 1 $S^L S^L$: 1 $S^L S^R$
 F_2 ratio is : 1(Round) : 1 (Oval).
20. (a) Splashed-white feathered chickens are incompletely dominant over Black-feathered chickens.
- (b) Suppose, Splashed-white feathered genotype— $F^S F^S$
 Black-feathered genotype— $F^B F^B$
 So, State-blue feathered genotype— $F^S F^B$.
21. Roan bull $\times$ Cows of Group-A (White) = 1/2 Roan + 1/2 White;
 Roan bull $\times$ Cows of Group-B (Red) = 1/2 Roan + 1/2 Red;
 Roan bull $\times$ Cows of Group-C (Roan) = 1/2 Roan + 1/4 Red + 1/4 White.
22. (a) From Cross 1, the 1:1 ratio suggests that short leg is heterozygous, showing incomplete dominance.
 (b) From Cross 2, the 2:1 ratio indicates that the short-leg gene is probably lethal when homozygous.
23. (a) The yellow strain is not pure breeding; yellow mice are heterozygous, probably showing absence of dominance.
 (b) The 2:1 ratio suggests the action of a lethal gene which gives yellow coat in the heterozygous state.
 (The yellow gene is termed y and its normal allele $+$)
- | | | | |
|------------|-------|----------|---------------|
| (Yellow) | $+y$ | $\times$ | $+y$ (Yellow) |
| Gametes : | $+ y$ | | $+ y$ |
| Offspring: | $++$ | $+y$ | yy |
- 1 Normal : 2 Yellow: 1 Dies
- (c) The yy offspring is never born; it dies *in utero* sufficiently early in development to be absorbed;
 (e) By dissection of the uteri of female yellow mice at intervals (5, 8 days, etc.) after mating with yellow males.
24. (a) 1/2
 (b) 2/3
25. (a) 3 Green : 1 White
 (b) The number seem to be reasonably close to the expected ratio but it is not possible to decide whether or not this amount of deviation from the expected ratio is significant without conducting a statistical analysis (i.e. Chi-square test, See *Experiment 10 in this Chapter*).
26. (a) Father, $Rh^+ Rh^-$
 Mother, $Rh^- Rh^-$
 First child, probably $Rh^+ Rh^-$
 Second child, $Rh^+ Rh^-$
 Third child, $Rh^+ Rh^-$
 Fourth child, $Rh^- Rh^-$.

230 Analytical Techniques in Biotechnology

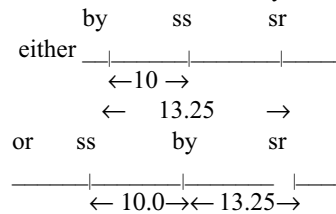
- (b) $\frac{1}{2}$.
- 27.(a) 3 Chinchilla : 1 Himalayan
(b) 2 Chinchilla : 1 Himalayan : 1 Albino
(c) 2 Full coat : 1 Chinchilla : 1 Himalayan
- 28.(a) The buck could be heterozygous with the Himalayan or the Albino allele, i.e. could be $c^{ch}c^h$ or $c^{ch}c$ and it would be best for him to mate the buck with a number of Albino does.
(b) The probability of being heterozygous is $(1/2)^6 = 1/64$, hence the probability of being homozygous = $1 - 1/64 = 63/64$, or 98.5% sure.
- 29.(a) 50%, S_1S_2 , S_1S_4
(b) 50%, S_2S_3 , S_3S_4
(c) 100%, S_1S_2 , S_1S_4 , S_2S_3 , S_3S_4
(d) 0
(e) 50%, S_2S_3 , S_3S_4

Experiment 6

30. Number of kinds of gamete = 2^n (where n = number of pairs of homologous chromosomes). Thus, 3 pairs of unlinked alleles would form 8 kinds of gamete. Since each kind of gamete listed forms approximately 1/8 of 400, the alleles are not linked.
31. If segregation were independent 1/8 of the gametes produced by the heterozygote would contain C, D and E genes. The fact that 146 (rather than the expected 50) offspring inherit C, D and E genes is evidence that the genes are linked.
- 32.(a) 10
(b) 1/10
(c) 9 ++ : 3 +bv : 3 bm + : 1 bmbv
(d) 3 normal : 1 brevis and brown midrib.
33. 10% 25% 22.8% 50%
AB = 45 AB = 37.5 AB = 38.6 AB = 25
ab = 45 ab = 37.5 ab = 38.6 ab = 25
Ab = 5 Ab = 12.5 Ab = 11.4 Ab = 25
aB = 5 aB = 12.5 aB = 11.4 aB = 25
- 34.(a) The genes bm and bv are linked, as are their wild-type alleles, of course.
(b) $\frac{+}{Bm} \frac{+}{bv} (\text{♀}) \times \frac{bm}{bm} \frac{bv}{bv} (\text{♂})$;
(c) 6%

$$(d) \frac{+}{bm} \frac{bv}{+} (\text{♀}) \times \frac{bm}{bm} \frac{bv}{bv} (\text{♂}).$$

- 35.(a) by and ss are linked, as are their wild-type alleles
(b) 10%
(c) 100 (♀) gametes Exp. %
by ss by + by + 45
(d) $\frac{+}{by} \frac{ss}{+} (\text{♂}) \times \frac{+}{by} \frac{ss}{+} (\text{♀})$ by ss 5
by ss by ss by + 5
by ss 45
- 36.(a) 13.25%
(b) Because sr and ss could be on the same or different sides of the by locus:



$$(c) \frac{ss}{+} \frac{sr}{+} (\text{♀}) \times \frac{ss}{+} \frac{sr}{+} (\text{♂})$$

- 37.(a) $\frac{ss}{+} \frac{sr}{+} \frac{ss}{+}$
← 10.0 → ← 3.5 →
(b) By carrying out a three-point cross instead of three two-point crosses. This would also have a clue about the possibility of double cross-overs between by and sr.

$$\frac{by}{+} \frac{ss}{+} \frac{sr}{+} (\text{♀}) \times \frac{by}{+} \frac{ss}{+} \frac{sr}{+} (\text{♂})$$

- 38.(a) COV b/pr = 57 + 3 out of 1000 = 6%
(b) COV pr/en = 72 + 3 out of 1000 = 7.5%

$$(c) \frac{b}{sc} \frac{pr}{+} \frac{en}{+}$$

$$39. \frac{48.5}{sc} \frac{54.5}{+} \frac{62.0}{+}$$

$$40. \frac{c}{C} \frac{sh}{sh} \frac{wx}{Wx} (\text{♀}) \times \frac{c}{c} \frac{sh}{sh} \frac{wx}{Wx} (\text{♂})$$

Experiment 7

41. 2/3
42. The animal in question must be female. Since $\frac{1}{2}$ of the male offspring would be expected to abort, the proportion of its surviving offspring expected to be males would be $\frac{1}{2}$.
(a) Half the female offspring would be carrier.
(b) No female embryo would abort.
43. (a) The female chicks are non-barred.
(b) (Cock) XX $\times$ X^BO (Hen)

XX ^B	XO
(Barred ♂ chick)	(Non-barred ♀ chick)
44. XX (♀) $\times$ X^BO (♂)

Gametes: All X	X ^B O
F ₁ offspring: XX ^B	XO

All ♀♀ F₁ are Bar-eyed and all ♂♂ are wild type.
45. (a) 2/3
(b) 1/3.
46. (i) True; the parents can donate aB and ab gene.
(ii) False; the male has only one X chromosome and therefore, cannot possess both B and b genes.
(iii) True; a black male would inherit aB genes from its mother.
(iv) False; black female would have two B genes but a yellow father would have none to give.
(v) True; she has both B and b genes to combine with whatever genes the male would donate.
47. Since the woman is heterozygous (inherits an X chromosome carrying a colourblind gene from her father) half her son would be expected to be colourblind and half normal sighted. All her daughters would be expected to be normal sighted but half of them would be expected to be carriers (heterozygotes).
48. Yes
49. (a) $\frac{1}{2}$
(b) $\frac{1}{4}$
(c) $\frac{1}{2}$
50. (a) Half the sons normal and half haemophiliac, half the daughters normal (but carrier) and half haemophiliac.
(b) Half the sons haemophiliac and half normal; none of the daughters would be haemophiliacs but half would be expected to be carriers.

51. The woman must inherit the gene from her father and is thus heterozygous. None of her daughters would be expected to show the disease but half of them would be expected to be carriers.

Half her sons would be expected to be haemophiliac. The probability of a child being haemophiliac = $\frac{1}{4}$

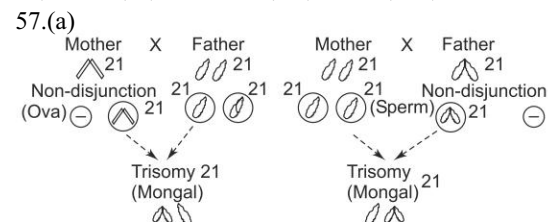
52. (a) Male (b) $\frac{1}{16}$
(c) $\frac{3}{4} \times \frac{3}{4} = \frac{9}{16}$ (d) $1 - \frac{9}{16} = \frac{7}{16}$.
53. (a) All ♀ offspring would be red-eyed. Half the ♂ offspring would be white-eyed and half red eyed.
(b) Possibly the virgins ♀♀ were not isolated soon enough and an F₁ cross had taken place.
54. Non-disjunction of sex chromosomes in either parent result in an XXY offspring with Bb genes for tortoiseshell fur pattern and a Y chromosome to give male features similar to Klinefelter's syndrome in man.

$X^B X^b \times X^b Y$ Non-Disjunction $X^B X^b Y$	$X^B X^b \times X^b Y$ or $X^b X^b Y$	$X^B X^b \times X^b Y$ Non-disjunction $X^B X^b Y$
--	---	--

55. (a) Mother: heterozygous for the colourblind alleles; (Father: carries the normal allele on his X chromosome);
Child : has only one sex chromosome, an X^b, which carries the colourblind allele that was inherited from the mother.

(b)	Father XY	$\times$	Mother X [*] X
	Non-disjunction		
	Gametes		X [*] X
	(XY) (—)		Child —X [*]

56.	XX	$\times$	XY	XX	$\times$	XY
	Non-disjunction		Non-disjunction		Non-disjunction	
	(—)	(XX) (X)	(Y)	(X) (X)	(XY) (—)	(—)
	XO	XXY	XO	XXY	XXY	XXY
	(Turner's)	(Klinefelter's)	(Turner's)	(Klinefelter's)	(Klinefelter's)	(Klinefelter's)



232 Analytical Techniques in Biotechnology

- (b) Since mongolism (Trisomy 21) is associated with the mothers' age it may indicate that non-disjunction during oogenesis is a more likely cause.

Experiment 8

58. (a) bbbb or b⁴
 (b) a²b²
 (c) ab³
 (d) a³b
59. (a) ab³ × ab³;
 $F_1 = (1/2 ab + 1/2 b^2)^2$
 $= 1/4 a^2 b^2 + 1/2 ab^3 + 1/4 b^4$
 (b) a³b × a³b
 $F_1 = (1/2 a^2 + 1/2 ab)^2$
 $= 1/4 a^4 + 1/2 a^3 b + 1/4 a^2 b^2$
 (c) a²b² × ab³,
 $F_1 = (ab)(1/2 ab + 1/2 b^2)$
 $= 1/2 a^2 b^2 + 1/2 ab^3$
60. (a) a³b × a³b
 $F_1 = (1/2 a^2 + 1/2 ab)^2$
 $= 1/4 a^4 + \dots$ Ans. 1/4;
 (b) $F_1 = (1/2 a^2 + 1/2 ab)^2$
 $= \dots 1/2 a^3 b \dots$ Ans. 1/2.
61. (a) 8''
 (b) 6''
 In this example a and b represent the alleles s and l and n = 6 (the number of alleles involved).
 Thus the distribution of offspring genotypes of a cross between 7'' plants ssslll × ssslll
 $= (s + l)^6$
 $= s^6 + 6s^5l + 15s^4l^2 + 20s^3l^3 + 15s^2l^4 + 6sl^5 + l^6$;
 Categories 1 + 6 + 15 + 20 + 15 + 6 + 1 = 64
 (c) 10'' variety is represented by l⁶
 $= 1/64$ of the offspring;
 (d) 7'' variety is represented by s³l³
 $= 20/64$ of the offspring;
 (e) The 5'' variety is represented by s⁵l
 $= 6/64$ of the offspring.

Experiment 9

62. Genetic variance = 0.897
 Phenotypic variance = 2.062
 Environmental variance = 1.164
 Heritability (Broad sense) = 43.52%

Low heritability value due to higher environmental variance and low genetic variance is an indication that the high yielding strains are not suitable for further improvement in wheat yield.

63. Genetic variance = 0.1165
 Phenotypic variance = 0.1515
 Environmental variance = 0.035
 Heritability (Broad sense) = 76.89%

Heritability (Broad sense) of 12 rice cultivars are due to high gene effects and low environmental variance. Rice cultivars may be useful for further improvement and breeding work.

Experiment 10

64. (a) 1/16 (b) 3/8
 (c) 1/4
65. The parents are obviously heterozygous, therefore, p_(brown) = 3/4 and p_(blue) = 1/4;
 $(3/4)^3(1/4) 4!/3! = 27/64$
66. (a) 1/100;
 (b) $(1/10 \times 4/9)^2 \cdot 1/4 = 1/2025$.
67. The probability of an individual producing a second, identical, gamete is $(1/2)^{23}$, thus, the probability of two such gametes combining to form an identical sibling is $(1/2)^{46}$, or about once in every 7×10^{12} children of a marriage.
68. (a) Yes; the χ^2 of 0.17 does not give a significant (less than 5% probability) value.
 (b) 60 – 80%.
69. From the table this χ^2 corresponds to a chance occurrence of about 1%, hence, this is a highly significant deviation from the expected result.
70. From the χ^2 table it is seen that a χ^2 of 5.7 for four classes gives a chance occurrence of between 10 and 20%, therefore, the deviation from the expected 1:1:1:1 ratio is not significant.
71. (a) Yes (b) 1/2R and 1/2r
 (c) Same answer as (b)
 (d)

♀ \ ♂	0.5R	0.5r
0.5R	0.25RR	0.25Rr
0.5r	0.25Rr	0.25rr

- (e) 1/4 RR + 1/2 Rr + 1/4 rr (Same as parental population)

72. (a) q (frequency of t allele) = 0.6
(b) $p = 0.4$
(c) Heterozygotes = $2pq$
 $= 0.48$
 $0.48 \times 25 = 12$ students.
73. (a) 0.5
(b) 50%
(c) $\frac{2}{3} \times \frac{2}{3} \times \frac{1}{4} = \frac{1}{9}$ or 11.1%
74. (a) 9/16 or 56.25%
(b) 6/16 or 37.5%.
75. About 9 per 10,000 births.

Experiment 10

1. First match to see whether U or T is present. If present, it will indicate that molecule is RNA or DNA, respectively. Therefore, molecule b is RNA, and a and c are DNA. If the base composition is compared, we find in the double-stranded molecules G pairs evenly with C and while A pairs evenly with T (or U). As such, b is a single stranded RNA, while a and c is double-stranded DNA.

As melting temperature increases with the amount of G-C, so the melting temperature of C will be greater than a.

2. (i) 26%G; 26%C; 24%A and 24%T.
(ii) Answer will be same, but 24%U and 0%T.

Explanation: As per Chargaff's rule the quantity of A = T. The sum total of all bases should be 100% i.e. $A + T + C + G = 100$.

As $G + C = 26 + 26 = 52\%$, then $(A + T) = 100 - 52 = 48$.

Since $A = T$, $\frac{1}{2}(48) = 24\%$ each, if the molecule is DNA, but in an RNA, where U replaces T, U will be 24%.

- 3.(a) A primosome = A helicase + A primase; it opens the DNA and creates RNA primers on Lagging strands and part of the replisome.

A replisome = A primosome + 2 copies of DNA polymerase III.

- (b) It coordinates replication on both the leading and lagging strands of the Y-junction.

4. (i) Complementary transcribed RNA strand for each DNA strand will be

For upper strand: CGAUGCCUAACGAC

For lower strand: GCUACGGAUUGCUG

If we look for AUG, it is present in the upper strand of transcribed RNA because polarity of the

start codon is 5'-AUG-3'.

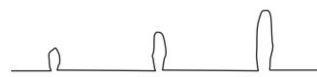
- (ii) As transcription starts $5' \rightarrow 3'$, and since nucleic acids are anti-parallel, therefore upper left end DNA strand will be $3'$ end.

5. The following will be the transcribed strand:

3'-GGTAGTACTGTCTGGGAACGATTGCG-5' ←

5'-CCATCATGACAGACCCTTGCTAACGC-3'

As U in RNA pairs with A in DNA and transcription proceeds $5' \rightarrow 3'$, the $5'$ end of the RNA is opposite the $3'$ end of the DNA.



6. There are four coding regions (exons) and three introns, represented by single-stranded DNA loops. The thick line represents RNA-DNA hybrids.
7. Partial digestion of molecule 2 leads to the following molecule:

AAAAAAAAAAAA

TTTTTTTTTTTT

Some of these molecules will form a circle with the single stranded Ts paired with single stranded As. The circle eliminates the free 5' phosphate, and the enzyme can no longer work.

8. Separately each enzyme produced two fragments, as such; the molecule has one site for each enzyme. Moreover, as each enzyme gives different sized fragments, the sites must be situated asymmetrically along the DNA.

$\begin{array}{ccc} \text{ecoRI} & & \text{bgl II} \\ 800 \downarrow 1200 & & 500 \downarrow 1500 \end{array}$

However, if *bg*III cuts in the 800 bp fragment of *eco*RI, we can expect to see 300, 500 and 1200 bp site cannot be 800 bp region of *eco*RI but in the other region i.e.1200 bp site.

*eco*RI *bgl*II

↓ ↓

800 700 500

- 9.

<i>eco</i> RI		<i>bam</i> II	
600	↓	850	↓
1400		1150	
1400		↓	
850	↑	550	↑
<i>bam</i> II		<i>eco</i> RI	

234 Analytical Techniques in Biotechnology

10. A one-base change in the codon for methionine (AUG), could result in following six amino acids.
 - (i) AUG changes to UUG or CUG by replacing A, results in Leu;
 - (ii) AUG changes to GUG, by replacing A, results in Val;
 - (iii) AUG changes to AAG, by replacing U, results in Lys;
 - (iv) AUG changes to ACG, by replacing U, results in Thr;
 - (v) AUG changes to ACG, by replacing U, results in Arg;
 - (vi) AUG changes to AUA or AUU or AUC, by replacing G, results in Ile.
11. (b) Lane 2
12. (e) All of the above are major classes
13. (e) Western blotting
14. (c) Peptidyl transferase
15. (a) P site

ANNEXURE VI

The table (below) of chi-square (χ^2) values shows the probability of chance occurrence as a percentage against the number of classes concerned.

In statistics it is customary to use the term “degree of freedom” to indicate the degree of latitude of choice which exists in a given situation. Thus, where two classes exist the degree of freedom is 1; where n classes exist, the degree of freedom is $n - 1$.

NUMBER OF CLASSES	PROBABILITY OF CHANCE OCCURRENCE IN PERCENTAGE								
	95%	90%	80%	60%	40%	20%	10%	5%	1%
2	0.004	0.016	0.06	0.25	0.7	1.6	2.7	3.8	6.6
3	0.1	0.2	0.5	1.0	1.8	3.2	4.6	6.0	9.2
4	0.4	0.6	1.0	1.9	3.0	4.6	6.3	7.8	11.3
5	0.7	1.1	1.7	2.8	4.0	6.0	7.8	9.5	13.3
6	1.1	1.6	2.3	3.6	5.1	7.3	9.2	11.1	15.1
Non-significant								Significant	

NOTE: Chi-square table showing the percentage probability of chance occurrence against number of classes.

ANNEXURE VII

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ANNEXURE VIII

Addresses of Some Suppliers of Equipments (Minor and Major), Chemicals, Glass- and Plastic-wares

1. M/S Bangalore Genei (P) Ltd., (Only for Bangalore Genei products)
No. 6, 6th Main, BDA Industrial Suburb,
Near SRS Road, Peenya,
BANGALORE-560 058.
Tel: 91-80-28391453; 28396894; 28378057
FAX: 91-80-28391622
E mail: genei@sanmargroup.com
Web: www.bangaloregenei.com
M/s BANGALORE GENEI (P) Ltd,
M-63, Lajpat Nagar II, NEW DELHI-110024.
Tel: 2632084; 2632101;
FAX: 91-011- 26320916
E mail: bgpl@del2.vsnl.net.in
2. M/s GENETIX Biotech Asia (Pvt.) Ltd.,
71/1, First Floor, Shivaji Marg, Najafgarh Road,
NEW DELHI -110 015.
Tel: 91-11-45027000
FAX: 91-11-28419631.
E mail: info@genetixbiotech.com
(For Fermentas, Cayman. Qiagen, Nalgene, Amresco products etc.)
3. M/s S.V. INSTRUMENTS ANALYTICA (P) Ltd.,
3269, Ranjit Nagar, Near Pusa Gate,
NEW DELHI-110 008.
Tel: 91-11-25843571; 25843572
E mail: svianalytica@vsnl.com
Web: www.svianalytica.com

4. M/s MERCK,
Merck Specialities (P) Ltd.,
Shiv Sagar Estate 'A', Dr. Annie Beasant Road,
Worli,
MUMBAI- 400 018.
Tel: 91-022-66609134/9133/9139
FAX: 022-24954590
E mail: chemical.info@merk.co.in
Web: www. Merk-chemical.com/in
(For Laboratory chemicals)
5. M/s BIORAD,
Biorad Laboratories (India) P. Ltd.,
Bio Rad House, Plot No. 86-87, Udyog Vihar
Phase IV,
GURGAON- 122 015. (HARYANA).
6. M/s LAB INDIA,
Lab India Instruments (P) Ltd.,
372, Udyog Vihar, Phase II,
GURGAON-122 016 (HARYANA).
7. M/s G. E. HEALTH CARE,
G. E. Healthcare Biosciences Ltd.,
No. 249 3rd Floor, Okhla Industrial Estate, Phase
III,
NEW DELHI- 110 020.
Tel: 91-011-4100192/22/23,
FAX: 91-011-41001928
Web: www.gelifesciences.com
8. M/s PERKIN ELMER (India) Pvt. Ltd.,
4A, Merlin Links, 166B, S. B. Mukherjee Road,
KOLKATA-700 026.
Tel: 91-033-24631517/1518/6641.
Fax: 91-033-24631519.
Web: www.perkinelmer.com
9. M/s TARSONS PRODUCTS (P) Ltd.,
856, Marshal House, 33/1, Netaji Subhas Road,
KOLKATA-700 001.
Tel: 91-033-22204022; 22200925; 22211013
Fax: 91-033-2221 1014.
E mail: tarsons@satyam.net.in
(For Pepetman, Plastic-wares)
10. M/s HiMEDIA LABORATORIES Ltd.,
A-406, Bhaveshwar Plaza, LBS Marg,
MUMBAI-400 086.
Tel: 25003747; 25000970; 25001607;
2500 0653
Fax: 91-022-25005764
E mail: info@himedialabs.com
11. M/s BOROSIL GLASS WORKS Ltd.,
Khanna Construction House,
44, Dr. R. G. Thadani Marg, Worli,
MUMBAI-400 018.
Tel: 91-022-24930362; 24930366.
Fax: 91-022-24950561.
M/s BOROSIL GLASS WORKS Ltd.,
19.90, Cannaght Circus, Madras Hotel Block,
NEW DELHI-110 001.
Tel: 91-011-23343897; 23742136; 23743732.
Fax: 91-011-23746689.
E mail: dsobora@ndf.vsnl.net.in
(For Glass-wares)
12. M/s QUALIGENS FINE CHEMICALS,
Glaxo Smithkline Pharmaceuticals Ltd.,
Dr. Annie Beasant Road, Worli,
MUMBAI-400 125.
Tel: 24933871; 24933514.
Fax: 91-022-24962573
E mail: inqfccustomer@care@gsk.com
13. M/s S.D. FINE-CHEMICAL Ltd.,
315-317, T. V. Ind. Estate,
248, Worli, MUMBAI-400 025.
Tel: 91-022-24936114; 24932429.
Fax: 91-022-24937232.
E mail: sdfcl@giasbm01.vsnl.net.in
14. M/s SISCO RESEARCH LABORATORIES
Ltd.,
2-F, Satyam Industrial Estate,
'C' Wing, 2nd Floor, Dr. Cardinal Gracious Road,
Chakala,
Andheri (E), MUMBAI-400 099.
Tel: 91-022-28203882
Fax: 91-022-28380998.
E mail: sri@bom3.vsnl.net.in

Index

A

Abscisic acid 127
Accident and Injuries 3
Acclimatization 118
Acetosyrigone 198
Acrocentric 37
Acrylamide 179
Additive affect 63
ADP-glucose 145
Adsorption 109
Aerobic 91
Aerosols 207
Agarose gel electrophoresis 172
Agranulocyte 182
Alcoholic fermentation 159
Alginate 140
Allele(s) 31
Anaerobic 91
Analysis of variance 68
Analysis of variation 66
Analyt 1
Analytical techniques 1, 178
Animal starch 145
Anionic detergent 178
ANOVA 68
Antibiotic 96
Antibody 213
Antigen 213
Antimicrobial 96
Antimicrobial chemotherapeutic agents 98
Antimicrobial chemotherapeutic chemicals 98
Antimycotic 207
Antiseptics 96, 98
Application of practical knowledge/experience 27

Aseptically 118
Aseptic technique 83
Asexual reproduction 31
Autoclaving 37
Autosomes 126

B

Back-cross 39, 41
Bacteriophage 109
Basal medium 114
Basic dye (Crystal-violet) 85
Basic pre-laboratory 2
Basophiles 182
Binary vector 198
Binomial Nomenclature 2
Biochemical reaction 91
Biochemical Test 91
Biochemical testing 91
Bio-degradable 102
Bio-informatics 202
Biology Lab safety rules 3
 accident and injuries 2
 basics 4
 biology labs do's and don'ts 4
 cleanliness 4
 clothing 3
 handling chemicals 3
 handling glassware and equipments 4
 handling heating substances 4
 handling microorganisms or cultures 4
Biotechnology 1
Black Bin 7
Broad-spectrum 98
Broth culture 89

238 Index

BSA 151
Bubble-point 29
Budlets 122

C

Callus 123
Callus index 123
Callus induction 121
Capsid 109
Capsomers 109
Carrier 58
Carrier matrices 155
Casein 92
Catalase activity 91
Catalyst 159
C-bands 44
Cell culture 118
Cell Cycle 33
Cell-suspension 54
Cellular 'glue' 208
Cellular organelles 176
Centimorgan 15
Centrifugation 27
Charles Chamberlain 41
Checker board 95
Chemical agents 5
Chemicals 105
Chemiluminescence immunoassay 211
Chemo-heterotrophs 96
Chemotherapeutic agent 96
Chemotherapeutic antimicrobial chemical 73
Chi-square 71
Chi-square test 39
Chi-square (χ^2) Test 73
Chloroplast 177
Chromatid 32
Chromatids 36
Chromatin 93
Chromogenic 91
Chromogenic 44
Chromogenic substance 165
Chromomere 36
Chromomeres 61
Chromosomal DNA 188
Chromosome Abnormality 96
Cidal 96
Cidal action 4
Cleanliness 3
Clothing 52

Co-cultivation 198
Coefficient of coincidence 158
Coliphages 196
Collagenase 208
Colloidal 78
Colony 78, 105
Columella 8
Competence 198
Competent cell 186
Conditions 63
Confluent culture 208
Continuous variation 53
Coupling phase 52, 155
Covalent bonding (of enzyme) 52
Cross Over Values 37, 54
Cryptic 84
Culture tube 8
Curriculum 107
Cyclosis 31
Cytokinesis 126
Cytokinin 127

D

Databank 203
Death-phase 89
Decomposition 159
Decontamination 96
Denaturation 17
Denaturation 157, 188
Denaturing enzyme 154
Desiccation 88
Dew-point 29
DH5 α strain 186
Differential expression 211
Differential media 78
Differential stain 85
Differentiation 118
Dihybrid 11
Dilutions 28
Direct sequencing 195
Disaccharide 153
Discrete colonies 78
Disinfectant 96
Disinfectants 98
Disinfection 96
Dissemination 81
Distillation 38
DMSO 207
DNA 170

DNA ladders 172
 DNA polymerase 175
 Dominance 49
 Dry heat 97
 Duplicate Factors 93
E
 Electricity 5
 Electrofusion 137, 138
 Electrophoresis 20, 172, 178
 agarose Gel 20
 Electrophorogram 203
 Embryo 140
 Embryogenesis 131
 Embryoids 122
 Embryo-rescue 121
 Encapsulation 140
 Endoenzymes 91, 92
 Endogenous 126
 Endosmosis 137
 Endosperm 140
 Endospore 88
 Enteric bacilli 78
 Environmental 66
 Environmental variance 68
 Enzyme 153
 Enzyme fractional distillation 159
 Eosinophil 182
 Epistasis 46
 Escherichia coli 186
 Essential elements 114
 Ethical Issues 5
 Ethidium bromide 169
 Ethylene 127
 Euchromatin 36
 Eukaryotes 36
 Evan's blue 108
 Exoenzymes 91, 92
 Exosmosis 137
 Expected 71
 Experimental evidence 1
 Explant 118
 Expression vectors 195
 Expressivity (or penetrance) 45
 Extraction buffer 168
 Eyepieces 13

F

Faculty 8

Fermentation 159
 Fermentation of Carbohydrate 91, 92, 94
 Fetal Bovin Serum (FBS) 207
 Filtration 96
 Fluid Volume 12
 Fluorescent diacetate (FDA) 108
 Folin-Ciocaltean reagent 151
 For keeping a 7
 Fractionation 149
 Froth 159
 Fusogenic chemical 137, 138

G

Gamete 31
 Gametogenesis 31, 32, 39
 GDP-glucose 145
 Gel tracking dye 172
 Gene cloning 195
 Gene pool 74
 General information 7
 General information for keeping a laboratory
 Notebook 7
 Genetic 66
 Genetic interaction 46
 Genetic mapping 52, 56
 Genetic parameters 66
 Genetic variance 68
 Genome 36
 Genomic DNA 168
 Genotypic ratio 39
 Genus 15
 G (Giemsa dark) bands 44
 Glycogen 145
 Gram negative 85, 86
 Gram positive 85
 Gram's decolourizer 85
 Gram stain 85
 Granulocyte 181
 Growth medium 114
 GUS-activity 198

H

Habituation 123
 Haemagglutination 215
 Handling chemicals 3
 Handling glassware and equipments 3
 Handling heating substances 4
 Handling microorganisms or cultures 4
 Hardening 114, 69

240 Index

Hardy Winberg's Law 74
 Heat-labile 114
 Heritability 66, 43
 broad 69
 broad sense 69
 narrow sense 70
 Heterochromatin 36
 Hetero-fermentor 161
 Hetero-karyon 137
 Heterokaryon 138
 Heterozygous 39
 Holandric 58
 Homogenization 176
 Homo-karyon 137, 138
 Homozygous 39
 Hot air sterilization 97
 Hydrolyse (or breakdown) 91
 Hydrolysis 153
 Hydrophilic 178
 Hypertonic 96
 Hypertonic environment 97
 Hyphae 105
 Hypostatic 49
 Hypotonic 96
 Hypotonic environment 97

I

Ideal laboratory 8
 Ideal laboratory conditions 8
 faculty 8
 laboratory environment 8
 updated biological curriculum* 8
 Immobilization 155
 Immunization 213
 Immuno-blotting 211
 Incinerated 83
 Incineration 97
 Incompatibility 121
 Incomplete dominance 45
 Incubation 78
 Independent assortment 39
 Inherent deficiency 121
 Inheritance factor 38
 Inheritance mechanism 31
 Inoculum 83
 Inquisitiveness 1
 Instructor 6
 Instrumentation 1
 Interference 52

Interphase 31
 Interpreting results 78
In vitro 118
 Isoenzymes 178
 Isolated plate 96
 Isotonic 97

K

Karyotype 36
 Karyotype analysis 37
 Kinetin 126

L

Laboratory direction 1
 Laboratory environment 8
 Laboratory notebook 1, 7
 Laboratory reports 7
 Lag-phase 89
 Lawn 109
 Law of dominance 39
 Law of segregation 40
 Leaf disc method 198
 Lethal alleles 45, 47
 Leukocyte 181
 Ligation 195
 Linkage 52
 Lipid 86
 Lipidomics 148
 Lipids 148
 Log-phase 89
 Louis pasteur 27
 Lymphocytes 182, 201
 Lysis buffer 150
 Lytic life-cycle 109

M

Macerozyme 138
 Macronutrients 114
 Magnifications 14
 Mannitol 138
 Manual counting 89
 Map distance 52
 Material Safety Data Sheets (or MSDS) 5
 Maturation 110
 Media enrichment 78
 Medium 83
 Meiosis 31
 Meiotic division 31
 Membrane filter 114, 118

Meristematic 31, 118
 Meristemoid 129
 Metabolic rate 123
 Metabolize 88
 Metacentric 37
 Methods of isolating 79
 combination selective and differential media 79
 differential media 79
 enrichment media 79
 isolated plate 80
 pour plate method 79
 selective media 79
 spin plate method 79
 streak plate method 79
 use of specialized media 79
 Metric length 12
 Metrizoate 201
 Micro-chromosomes 44
 Microorganism 78
 Microscope 32
 Mitochondria 176
 Mitosis 31
 Mitotic division 31
 Mitotic index (MI) 32, 46
 Modified ratio 48
 Modified ratios (or genetic interaction) 39
 Moist heat 97
 Monocyte 182
 Monohybrid 39
 Monohybrid cross 46
 Morphogenesis 114
 Multiple alleles 50, 51
 Multiple allelic series 36
 Multi-shoots 121
 Murashige and skoog 115
 Mutation 105, 203
 Mycelium 105
 Mycology 105
 Mycoplasma 207
 Mycotic infection (or mycoses) 105

N

N 179
 Naked embryo 133
 Naked virus 109
 Narrow-spectrum 98
 Neutrophil 182
 N-methylebisacrylamide (bis 179

Non-degradable 102
 Non-disjunction 58, 61
 Non-pathogenic moulds 105
 aspergillus 106
 penicillium 105
 phialides 106
 rhizopus 106
 saccharomyces 106
 Normal distribution 66, 67
 Nucleocapsid 109
 Null hypothesis 71

O

Observed 71
 Optical Density (O.D.) 89, 167, 174
 Orcinol 165
 Organ culture 121
 Organic supplement 114
 Organogenesis 129, 131
 Osmo-regulator 137
 Osmotic lysis 85
 Osmotic pressure 97
 Osmoticum 137
 Over-lapping 63

P

Parasite 105
 Parenchyma 118
 Parental genotype 39
 Partitioning of variance 66
 Pasteurization 97
 Pathogenic 105
 Pattern of inheritance 31, 32
 PBMC/PBMNC 201
 PCR 193
 Pectolytic (or protolytic) enzyme 161
 Pellicle 89
 Penetration 110
 Peptidoglycan 85
 Permeable 107
 Phage typing 109
 Phenotypic 66
 Phenotypic classes 63
 Phenotypic ratio 39
 Phenotypic variance 68
 Philosophical and 9
 Philosophical and ethical issues 17
 Physical agents 95

242 Index

Physical basis 71
 Pigmentation 93
 Pipetman 48
 Plantgrowth regulators 126
 Plant hormones 126
 Plant propagules 140
 Plaque 110, 196
 Plaque-count 109, 110
 Plasma membrane 107
 Plasmid 186
 Plasmid DNA 188
 Plasticity 114
 Plate culture 83, 84
 Pleiotropic 63
 Pleiotropic effects 63
 Polyacrylamide gel 178
 Polygenic traits 71
 Polymerase Chain Reaction (PCR) 71, 193
 Polymerization 178
 Polymorphism 203
 Polymorphonuclear cells (PMN) 181
 Positioning the Slide 78
 Pour plate 41
 Precipitation of intracellular protein 188
 Preparations 129
 Primordium 92
 Probability 91
 Probability hypothesis 92
 Prokaryotes 118
 Proliferating 140
 Propagules 92
 Protease 133, 150
 Protease inhibitors 158
 Protein 205
 Proteinase K 170
 Protein hydrolysis 134, 137
 Proteolysis 150
 Proteolytic enzyme 208
 Proteome 138
 Protocorm 137

Q

Q-bands 44
 Quantitative or polygenic inheritance 63

R

Radiation 98
 ionizing 98
 ultraviolet 98

R-bands 52
 RBC or erythrocyte 181
 Recessive 52
 Recombination 7, 31
 Red bin 15
 Reduction division 53
 Regeneration 114
 Regulator 138
 Regulatory sites 195
 Reinfection 110
 Relative centrifugal force 52
 Release 110
 Replication 110
 Repulsion phase 15, 33
 Resting phase 172
 Restriction digestion 172
 Restriction endonuclease 172
 Restriction enzyme 174
 Restriction enzyme digestion 195
 Reverse transcriptase 165, 193
 RPMI 1640/DMEM 207

S

Safety measures for hazardous substances 5
 chemicals 5
 electricity 5
 Material Safety Data Sheets (or MSDS) 5
 ultraviolet Light 5
 Safety rules 1
 Samplers 81
 Sampling error 71
 Sanitizer 96, 98
 Saprophytes 105
 Sauerkraut 161
 SDS 178
 SDS-PAGE 179
 Secondary constriction 36
 Secondary sexual characters 58
 Sediment 89
 Segregation 39
 Selective combination 78
 Selective media 78
 Semi-solid medium 123
 Septa 105
 Sex-chromosome 36
 Sex determination 59
 drosophila 59
 in birds 59

- in flowering plants 59
- in insects 59
- Sex expression 61
- Sex-influenced genes 58, 62
- Sex-limited genes 58, 62
- Sex linkage 60
- Sex-linked gene 58
- Sexually reproducing 31
- Shoot primordial 121
- Significance 71
- Silica gel 149
- Size marker 172
- Skim milk 154
- Software 205
- Somatic cell fusion 137
- Somatic embryogenesis 131
- Somatic embryoids 121
- Sorbitol 138
- Species 15
- Spectrophotometer 10, 96
- Spectroscopy 10
- Spin plate 78
- Sporulation 88
- Stages of embryogenesis 131
- Standard deviation 66, 67
- Starch Hydrolysis 92
- Static 96
- Static action 96
- Stationary-phase 89
- Sterile distilled water 114
- Sterile medium 83
- Sterilents 135
- Sterile nutrient 83
- Sterilization 25
 - aseptic culture of microbes 26
 - broth culture 26
 - browning 26
 - chemical 25
 - heat (dry or wet) 25
 - inoculating loop 26
 - inoculum 26
 - laminar air flow 27
 - plant tissue culture 25
 - plate culture 26
 - ultra-filtration 25
- Stock solution 114
- Streaking 78
- Streak plate 78

- Sub-culture 118
- Sub-metacentric 37
- Suggestions to the 6
- Suggestions to the Instructor 6
- Super-coiled plasmid DNA 188
- Surfactant 135
- Suspended particles of proteins 157
- Symbiotic germination 133
- Synthetic seeds 140

T

- Taq DNA polymerase 193
- T-DNA 198
- Telocentric 37
- Testa 140
- Test cross 41
- Test-cross 39
- Test of Significance 73
- Thin Layer Chromatography (TLC) 148
- T. H. Morgan 54
- Totipotency 114
- Transcription 43
- Transformation 186, 197
- Transformation protocol 123
- Transformed bacterial cell 188
- Transgenic 197
- Transgressive variation 65
- Trans-illuminator/gel doc system 172
- Transmission 81
- Trichomes 135
- Trizol reagent 174
- Trypan blue 201
- Trypsin 208
- Trypsin (or collagenase) 208
- Turbidity 89

U

- Ultraviolet light 5
- Updated biological 8
- Uridine Diphosphate Glucose (UDP-glucose) 145
- UV-light Box 172
- UV-Spectrophotometer 89

V

- Variance 67
- Vector 194
- Viability 107
- Viral specificity 110
- Vir genes 198

244 *Index*

W

WASH 159

WBC 181

Western blotting 178, 211

White blood cells (WBC) 170

X

X or Y chromosome 58

Y

Yeast 159

Yellow bin 7

Plate I

With the help of scissors cut out all the chromosomes, match the pairs, paste them on your Practical Record according to the chromosomal Groups and indicate male/female in the Result



Plate-II

With the help of scissors cut out all the chromosomes, match the pairs, paste them on your Practical Record according to the chromosomal Groups and indicate male/female in the Result

