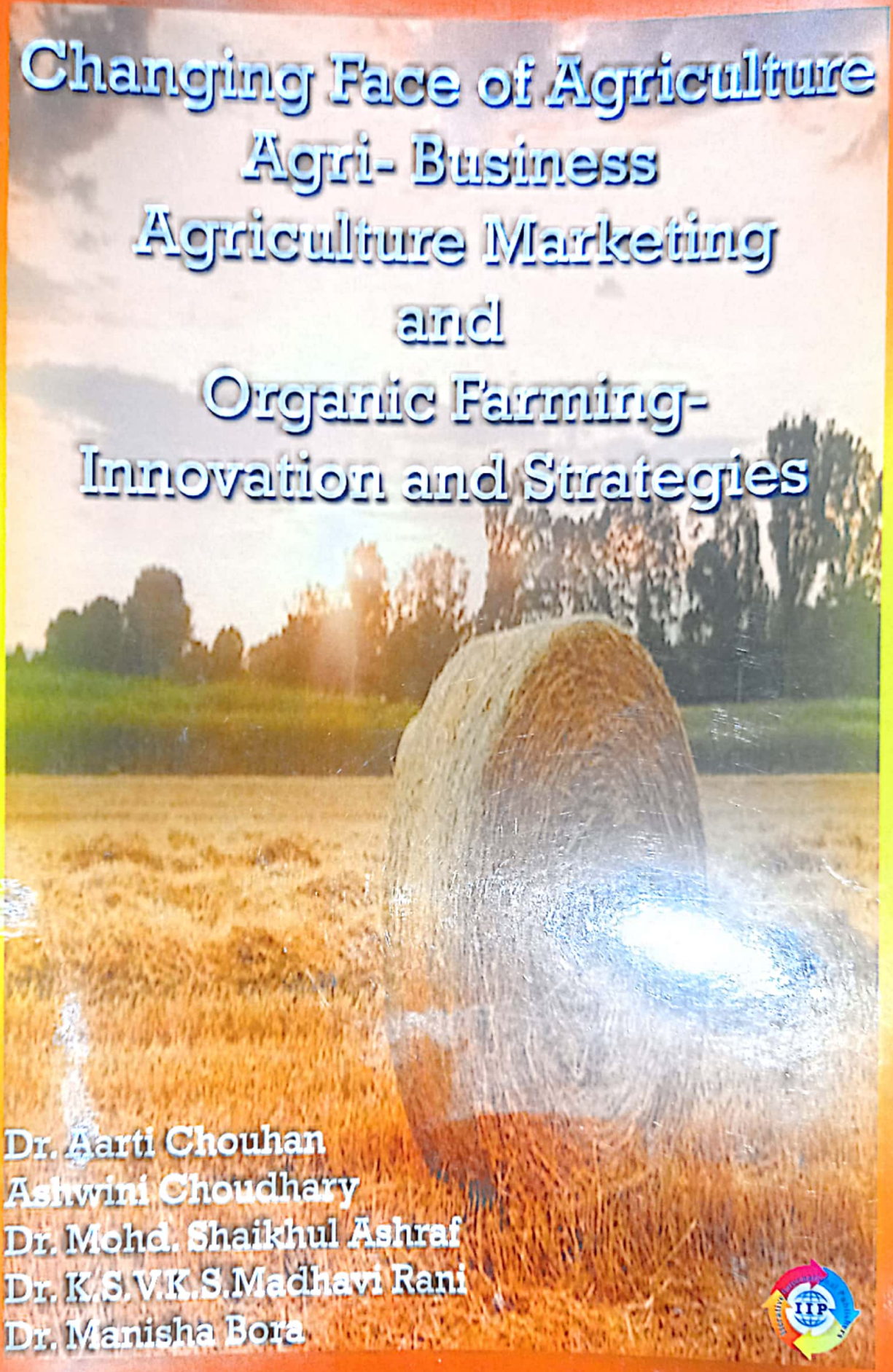
Changing Face of Agriculture Agri- Business Agriculture Marketing and Organic Farming- Innovation and Strategies



Dr. Aarti Chouhan
Ashwini Choudhary
Dr. Mohd. Shaikhul Ashraf
Dr. K.S.V.K.S.Madhavi Rani
Dr. Manisha Bora

Changing Face of Agriculture, Agri- Business, Agriculture Marketing and Organic Farming- Innovation and Strategies

First Volume

Editors

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First Volume - 2022

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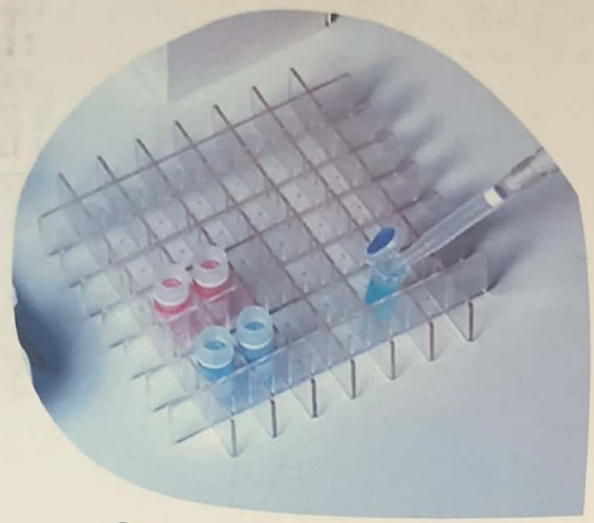

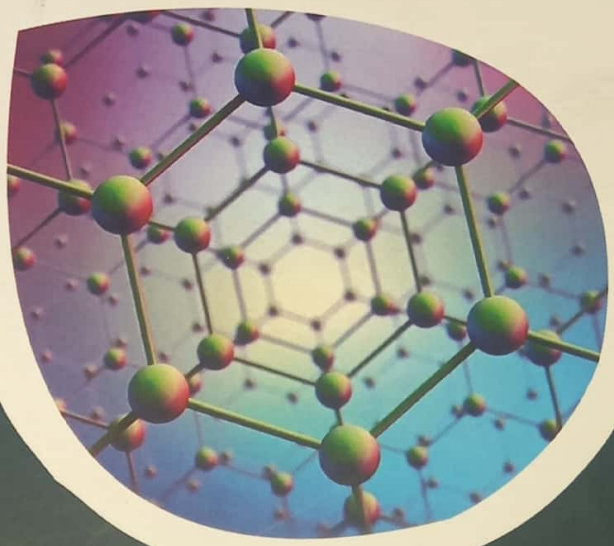

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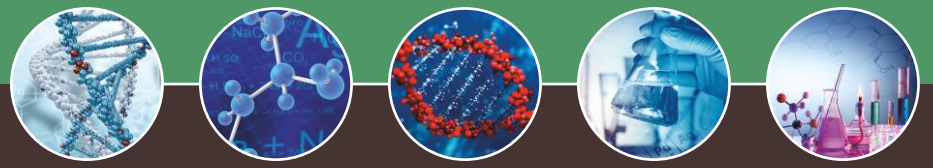

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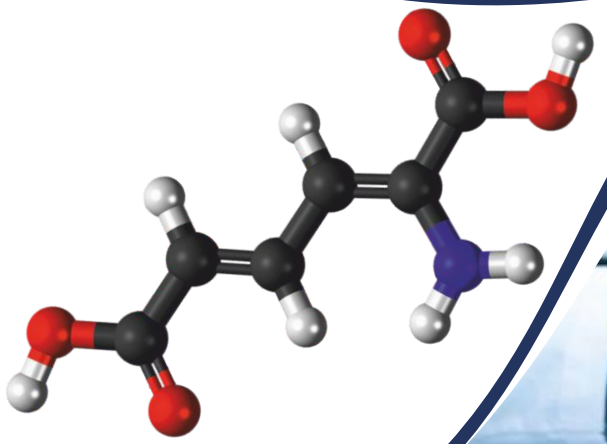

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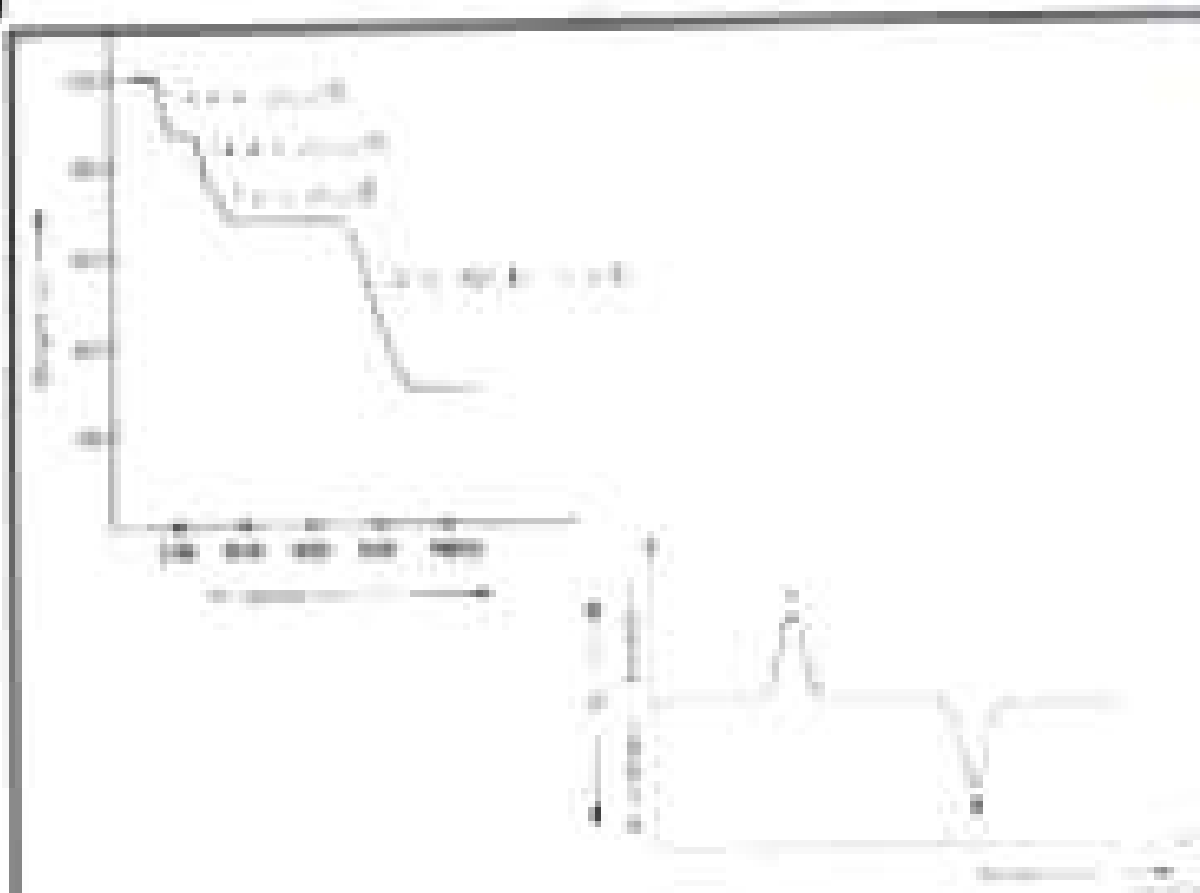

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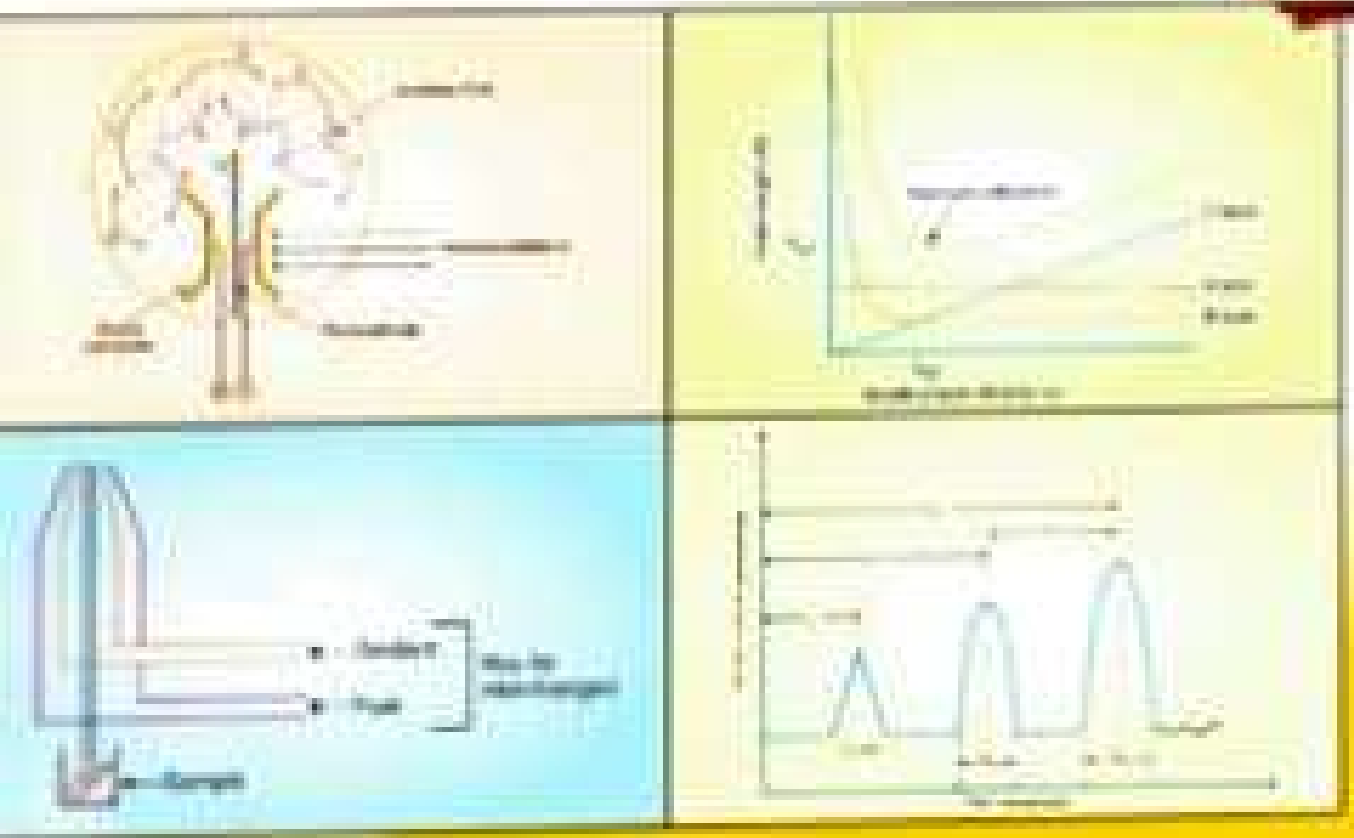

Practical Handbook on

Metabolism and Molecular Biology

MB 368 (T.Y B.Sc Microbiology)

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To add, he has following specifications: Research-gate Score: 14.98; h-index = 7; i-10 index = 4; Google Scholar Citations = 131; Publications: >43 (all in peer reviewed journals), Chapters= 5; Book = 3, IPR = 25; Patents = 18 (Published = 12), Design Registrations = 7, Scientific Projects Handled = 5; Awards = > 13; Guest Talks = > 20; Organizing Secretary for Scientific events => 9Scientific Projects Handled = 5; Awards = > 10; Guest Talks = > 18; Organizing Secretary for Scientific events => 8

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Introduction

Dear students, there are several experiments in Microbiology which are based on theoretical aspects. Basic knowledge of laboratory work is important for designing future research plan. It is a very crucial for the students that they should learn research skills. However, there are some difficulties that are observed in the minds of students during a scientific demonstration of the practical courses due to lack of detailed protocols. As a result, we have made sincere efforts to solve the problem in question through this book and provided up-to-date basic information and experiments based on theoretical knowledge. We are sure that the book will be valuable for students to get up the mark in scientific knowledge.

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This is to certify that Mr./Mrs./Dr./Miss ---------- -------

---------- has successfully completed the practical course entitled ----------

---------- on ----------

Signature of Course Teacher/ Internal Examiner	**Signature of Head of Department**	**Signature of External Examiner**

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Practical 1

CLINICAL BIOCHEMISTRY

Introduction:

The clinical tests are carried out in the laboratory for the diagnosis and management of disorders in patients.The indications or reasons for ordering laboratory management and for examinations are To confirm a clinical impression and diagnosis.

1. To rule out disorders.
2. To monitor the proper therapy.
3. To define the prognosis of the disease,
4. To screen out or to detect the proper stage of the disease.

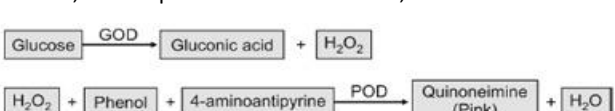
The biochemical pathways are very complex and analysis of every component is not possible. The analysis and measurement of these components indicate the possible maladies of the system. The changes due to any disease or imbalance in the metabolism are reflected in body fluids. e.g., blood, urine and CSF.

ESTIMATION OF BLOOD GLUCOSE

Name of the method: GOD-POD

Principle:

Hydrogen peroxide (H2O2) is released when glucose oxidase (GOD) converts the particular substrate -D- glucose to gluconic acid. Nascent oxygen (O2) is produced by the peroxidase enzyme when it reacts with hydrogen peroxide, and it subsequently combines with 4-amino antipyrine as well as phenol to develop the red quinoneimine color. The plasma glucose concentration seems to be directly proportional to the color intensity.Green or 530-nanometer colourimeters are used to measure color intensity, which is then compared to a standard specimen. Likewise,if not exposed to direct sunshine, the ultimate color will only be



sustainable for two hours.

Reagents:

1. GOD-POD Reagent
2. Glucose standard 100 milligram/deciliter& 500 milligram/deciliter
3. Test glucose

Protocol

1. Label the dry & clean tubes as follows: -
   Tube 1: blank, Tube No. 2: Standard I (SI), Tube 3: Standard II (SII), Tube 4: test glucose (TG), Tube 4: test sample (TS)
2. Perform the additions as shown in the table below.
3. The contents from all tubes should be mixed thoroughly.
4. 10 minutes of water bath incubation at 37 °C.
5. Mix properly.
6. Note down the absorbance of all standard, test & blank reactions at 505nanometers or with a green filter.
7. Calculate the concentration of glucose in the test & test sample by the given formula.

Preparation:

Description	Blank	Standard I	Standard II	Test Glucose	Test Sample
GOD-POD Reagent	3.0 millilitr	3.0 milliliter	3.0 milliliter	3.0 millilitr	3.0 milliliter
Standard 100 milligram/deciliter		30ul			
Standard 500 milligram/deciliter			30 ul		
Test Glucose				30 ul	
Test Sample					30 ul
Distilled water	30 ul				

To 100milliliter of GOD-POD reagent add 1.7milliliter of phenol. Prepare fresh

Observation:

Calculation:

Glucose (milligram/deciliter) in test glucose

$$= \frac{\text{Absorbance of TG} - \text{Absorbance of B}}{\text{Absorbance of SI} - \text{Absorbance of B}} \times 100$$

Glucose (milligram/deciliter) in test Sample

$$= \frac{\text{Absorbance of TS} - \text{Absorbance of B}}{\text{Absorbance of SI} - \text{Absorbance of B}} \times 100$$

Where,
100= Standards
SI= Standard 1
SII= Standard 2
TG= Test Glucose
TS= Test Sample

Result:

Conclusion:

Inference:

References:

1. Trinder P. 1969. Ann. ClinBiochem. 624
2. Tietz N. W. 1982. Fundamentals of Clinical Chemistry, 2ndEdn W. B. Saunders Co. Toronto

1.2 ESTIMATION OF BLOOD UREA

Name of the method: DAM (Diacetyl Monoxime)

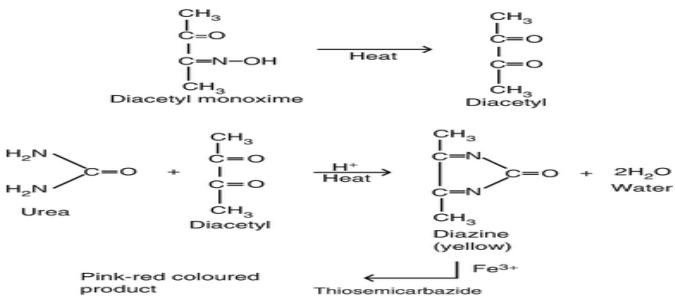
Introduction:

The body's primary byproduct of protein metabolism is urea.The liver produces several enzymes by which amino acids are removed from urea. The concentration of urea in the blood is the balance between the urea formation from the protein metabolism and the urea excretion by the kidney. A small amount of urea is lost in the faeces and serum. The normal value is 20-40milligram\ Determination of blood urea is important not only in many diseases of kidneys but in a wide range of conditions which are not primarily renal.

Determination of blood urea is important not only in many diseases of kidneys but also in a wide range of conditions which are not primarily renal. Raised blood urea is one of the commonest abnormal findings. Blood urea causes acute and chronic nephritis, hepato-renal syndrome and uremia due to a fall in glomerular filtration and also after a major operation (due to increased protein catabolism). The obstructions are due to an enlarged prostate gland affecting the flow of urine which causes fever, renal diseases, thyrotoxicosis, diabetic coma, Leukemia etc. There are so many conditions in which blood urea may be raised so the estimation has little value if perform as random diagnostic procedures. However, the estimation is valuable to note the progress of the case. Low blood urea sometimes occurs in late pregnancy in acute hepato-treatment. There are various methods available for determining blood urea level e.g., Urease, Nesslerization method, Diacetyl Monoxime Method etc.

Principle:

Thiosemicarbazide is present when hot, acidic Diacetyl Monoxime interacts with urea to generate a complex that is rose-purple in color, which may be detected by a calorimeter.



Advantages:

1. It is an easy, quick as well as one step process
2. De-proteinization is not required.

Sample:

- 0.02milliliter Serum or plasma. (Do not use anticoagulants containing Ammonium salts)
- Urine: Dilute 1:20.

Reagents:

Reagent 1: Urea Reagent.

Reagent 2: Diacetyl Monoxime (DAM).

Reagent 3: Working urea standard. (30milligram)

Preaparation of working soutions:

Solutions 1: Dilute 1 milliliter of reagent 1 to 5 milliliter with distilled water. Reagent 2 & Reagent 3 (standard) are ready to use.

Precautions:

1) Utilize dry glassware cleaned with chromic acid
2) Before using, let all solutions come to room temperature.
3) For each series of determinations, create a standard.
4) Because the procedure is extremely sensitive, the standard sample needs to be precisely quantified.
5) Before beginning the estimation, correctly label the test tubes as Blank (B), Standard (S), and Test (T), as the marks may rub off when the tubes are put in a bath of boiling water.

Calculations:
1.Serum /Plasma

$$\boldsymbol{Urea\ concentratio}\ (\boldsymbol{mg/dl}) =$$

O.D.of Test _____ X Standard Concentration O.D.of Standard

Blood Urea Nitrogen (milligram/deciliter)=Serum Urea (milligram/deciliter) × 0.4671

Normal Values:
Serum Urea: 0-40 milligram/deciliter
BUN: 10-20 milligram/deciliter
Urine urea: 20gm/liter

Protocol:

Description	**Blank**	**Test**	**Standard**
Reagent 1	2.5 milliliter	2.5milliliter	2.5milliliter
Test sample		0.01 milliliter	
Reagent3 (Working urea std 30 milligrams)			0.01milliliter
Reagent2 (Diacetyl monoxm ine)	0.25milliliter	0.25milliliter	0.25milliliter
Description	Blank	Test	Standard
Reagent 1	2.5 milliliter	2.5milliliter	2.5milliliter
Test sample		0.01 milliliter	
Reagent3 (Working urea std 30 milligrams)			0.01milliliter
Reagent2 (Diacetyl monoxm ine)	0.25milliliter	0.25milliliter	0.25milliliter

Observation:

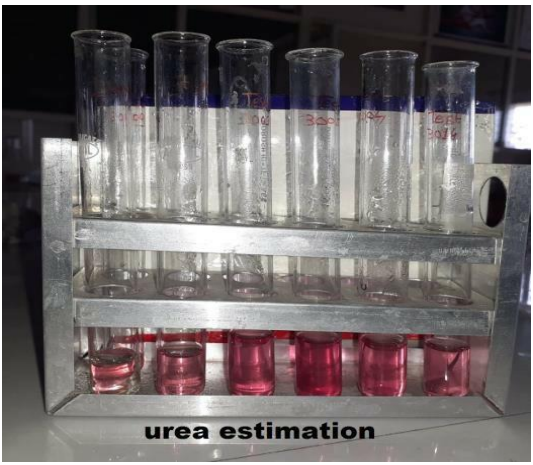


Figure: One of the steps involved in urea estimation process.

Result:

Conclusion:

1.3: ESTIMATION OF SERUM CHOLESTEROL

Introduction:

The major lipid present in the blood, brain, as well as bile, is cholesterol. Through blood (plasma) circulation Cholesterol from the liver is transported to various cells of tissues by low-density lipoproteins, very low-density lipoprotein and chylomicrons. If needed cholesterol is synthesized by the cells. The excess cholesterol is transported back to the liver by HDL. It is necessary for the synthesis of cellular membranes and steroids. In hypercholesterolemia, hyperlipidemia, nephritic syndrome, uncontrolled diabetes, and cirrhosis, the level is elevated. Reduced levels are seen in liver disorders, anemia, hyperthyroidism, starvation, and malabsorption.

Principle:

In the action of the enzyme cholesterol esterase, the cholesterol esters are hydrolyzed to release free cholesterol (CE). Cholesterol oxidase (CO) then converts the free cholesterol to cholestene 4-en-3 one while simultaneously producing hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide combines with 4AAP and phenolic chemicals to produce a colored complex that can be detected at 505 nanometers.

Reaction:

Cholesterol Esters $\xrightarrow{CE}$ Cholesterol + fatty acids

Cholesterol +O2 $\xrightarrow{Co}$ Cholesterol- 4en-3-One +H_2O_2

$2H_2O_2$+4AAP+Phenolic compound POD $\longrightarrow$ Quinonimine dye +$4H_2O$

Reagents:

1. Fresh clear serum or plasma (EDTA)
2. Cholesterol standard
3. Cholesterol reagent

Protocol:

Additions	Blank	Standard	Test
Cholesterol reagent	1.0 milliliter	1.0 milliliter	1.0 milliliter
Cholesterol standard	-	10ul	-
Sample(test)	-	-	10ul

5 minutes of mixing and incubation at 37 °C After 5 minutes, compare the absorbance of the test and the standard to a reagent blank using a 505-nanometer or green filter.

Normal Values:

Cholesterol: 140 to 260 milligram/deciliter.

Observation:

Calculation:

Cholesterol (milligram/deciliter) = Abs T: Abs SX200

Result:

Conclusion:

Reference:

1.Allain, CC, Poon, L, Clin. Chem. 20: 470(1974)

2.. I.F.C.C- Clin. Chim. Acta 87/3:459F(1978)

1.4 ESTIMATION OF ALBUMIN

Introduction:

Albumin consists of approximately 60% of the total proteins in the body, and the remaining is a major part of globulin. It is synthesized in the liver and maintains the osmotic pressure in the blood. Albumin also helps in the transportation of drugs, hormones and enzymes. Elevated levels are rarely seen and are usually associated with dehydration. Decreased levels are seen in liver diseases (hepatitis, Cirrhosis). Malnutrition, kidney disorders, increased fluid loss during extensive burns and decreased absorption in gastrointestinal diseases.

Principle:

Albumin binds with the dye bromocresol green in a buffered medium to form a green color complex. The intensity of the color formed is directly proportional to the amount of albumin present in the sample.

Reaction:

Albumin+ Bromocresol Green ⟶ Green Albumin BCG Complex

Reagents:

1. BCG reagent
2. Albumin standard(4g/dl)
3. Sample

Additions	Blank	Stanard	Test
BCG reagent	1.0 millilit er	1.0millilit er	1.0mill iliter
Distilled water	0.01mill iliter	-	-
Albumin standard	-	0.01milliliter	-
Sample(tes t)	-	-	0.01m illiliter

Mix well and incubate at 37°C for 5 min. Read the absorbance of the test (T) and standard (S) after 5mins at 630nanometer and compare with reagent blank.

Observation:

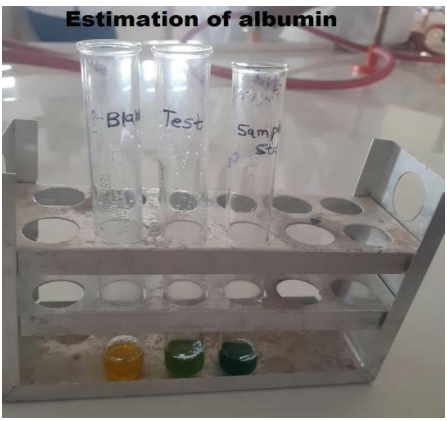


Figure: One of the steps involved in the albumin estimation process.

Calculation:

$$\text{Albumin in g/dl} = \frac{\text{Abs.T} \times 4}{\text{Abs.S}}$$

Result:

Conclusion:

1.5 Estimation of Protein:

Introduction:

Proteins are constituents of muscles, enzymes, hormones and several other keys functional and structural entities in the body. They are involved in the maintenance of the normal distribution of water between blood and tissue. Consisting mainly of albumin and globulin the ratio of these varies independently and widely in disease. Decrease levels are found mainly in malnutrition, impaired synthesis, and protein losses such as haemorrhage or excessive protein catabolism.

Principle:

Proteins in an alkaline medium bind with the cupric ions present in the biuret reagent to form a blue-violet color complex. The intensity of the color formed is directly proportional to the number of proteins present in the sample.

Reaction:

Protein + Cu^{++} ⟶ Blue violet color complex.

Reagents:

1 Biuret reagent

2 Protein standard (8g/dl)

3 Sample

Additions	Blank	Standard	Test

Biuret reagent	1.0 militers	1.0millilite r	1.0milliliter
Distilled water	0.02millil iter	-	-
Protein standard	-	0.02milliliter	-
Sample(test)	-	-	0.02millilit er

Mix & incubate at 37°C for 10min or at room temperature for 30min.

Read the absorbance of the test (T) and standard (S) after 5 min at 550nanometer and compare with the reading of blank.

Normal values :

Serum &plasma : 6 to 8g/dl

Observation:

Calculation:

Total Protein in g/dl = $\frac{\text{Abs.T}}{\text{Abs.S}}$ X 8

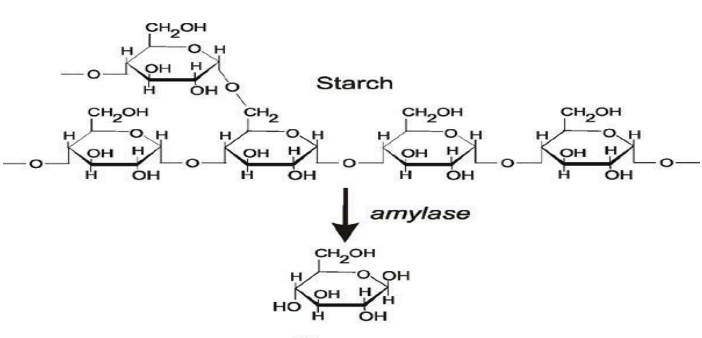
Result:

Conclusion:

Parctical 2- Enzyme production, purification, quantification and Immobilization

Introduction:

Amylases are starch-degrading enzymes. Bacteria (species of Bacillus) and fungi (Aspergillus, Rhizopus, Mucor) produce amylases, however, the enzymes from these two sources are not identical. Starch is made up of amylase and amylopectin. The enzyme alpha (α) amylases hydrolyse the α-1,4 linkage of starch in a random manner producing a mixture of D-glucose and dextrin. While beta (β) amylase hydrolyses alternate α-1,4 glycosidic bonds from the non-reducing end of the starch molecule, yielding the disaccharide, maltose.



2.1 Production of amylase

Laboratory level production of amylase from bacteria *Bacillus subtilis*.

Requirements :

1. Sterile Starch agar plates
2. Gram stain.
3. Starch agar broth
4. Nutrient broth containing 1% Starch
5. Ammonium sulphate
6. Phosphate buffer.

Protocol:

1. Keep starch agar plate air exposed for 5min and incubate at RT for 24hrs.
2. Pour Gram stain and observe the zone of clearance around the colonies against dark blue background.
3. Subculture the colony and perform the biochemical analysis to use this culture for amylase production.
4. Inoculate the culture in sterile nutrient broth with 1% starch. Take 10milliliter of cell suspension of *Bacillus subtilis* in 100milliliter of Starch agar broth (liquid medium in 250milliliter flasks) or 10milliliter of cell suspension subtilisin along with 100milliliter nutrient broth containing 1% starch in 250milliliter flasks.
5. Keep all the flasks on a rotary shaker at 150 rpm at room temperature for 48 hours
6. After 48 hrs. the flask was taken off the shaker. For *Bacillus*, the broth was centrifuged at 10,000 rpm. And by decanting the supernatant, cell-free broth (CFB) was obtained.
7. Using 5milliliter of broth at 20%, 40%, 60%, and 80% saturation, ammonium sulfate precipitation of CFB aliquots was done.

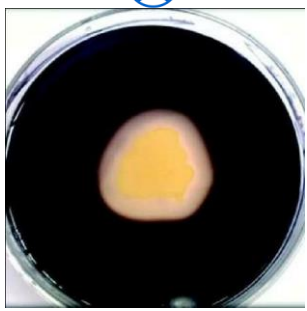


Table 1. Ammonium Sulfate Fractionation Table showing the mass (in grams) of solid ammonium sulfate to add per liter of solution at 20 °C. This table is adapted from *Protein Purification: Principles and Practice* 2nd Ed. (1987) Scope RK.

	Final Percent Saturation to be Obtained																
	20	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**	**65**	**70**	**75**	**80**	**85**	**90**	**95**	**100**
Starting % saturation	Amount of ammonium sulfate to add (grams) per liter of solution at 20 °C																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	357	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	292	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	227	265	304	344	386	429	475	522	571
30			0	30	61	92	126	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	97	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	266
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

2.2 PRECIPITATION OF AMYLASE:

Decreases in the hydration of protein in favor of neutral salt are observed when protein precipitation is obtained by using a high concentration of ammonium sulfate. Depending on the charge of the protein, the hydration layer surrounding the surface of the protein is transferred to the ions of the salt. The concentration required to precipitate a specific protein dependent upon the function of pH, temperature and salt used. The nature of salt, whether it is added as a finely ground powder or as a saturated solution of ammonium sulfate, the rate at which the salt is added (2- 10 g/min) and the length of time the suspension is stirred also affects the reproducibility of protein precipitation. Since the volume of the sample doubles when 50%

saturation is reached, for a small volume, the use of the saturated solution of ammonium sulphate is gentler on the protein.

Methods:

1. Ammonium sulphate should be added in small amounts. To achieve the required saturation, mix well the contents of the tubes.
2. After the addition of ammonium sulphate, keep the tubes in the refrigerator at 10^0C for 24hrs. (Standing periods can be 1.5 hrs to overnight)
3. After allowing tubes to stand overnight, the precipitate would be observed in some of the tubes.
4. The broth then should be centrifuged at 10000 rpm for 20 min at 40 C.
5. The supernatant and pellet (precipitate) were separated. The pellet was dissolved in 5milliliter of 0.02M phosphate buffer (pH 7.0)
6. Amylase activity and protein content (Folin- Lowry method) were checked in both the supernatant and the precipitate (suspended in a fixed aliquot of buffer).

2.3 DETERMINATION OF SPECIFIC ACTIVITY OF CRUDE AND PURIFIED AMYLASE

Protocol for amylase assay: -

1. 1 milliliter of the test sample, enzyme control, substrate control and reagent blank respectively were made according to the following scheme: -
2. The tubes containing the reaction mixture were kept at 37^0 c for 10 min.
3. 1 milliliter of DNSA reagent was added to each tube.

4.The tubes were kept in a boiling water bath for 10 min.

5.The solution was cooled and diluted to 10milliliter with distilled water.

6.The absorbance of the samples was read at 530nanometer.

To be added	Test Sample	substrate control	Enzyme Control	Reagent Blank
Enzyme	0.1	-	0.1	-
Substrate	0.9	0.9	-	-
Distilled water	-	0.1	0.9	1
Total volume	1	1	1	1

Observation:

Calculation:

Enzyme activity (mM of maltose/milliliter/min)

$$= \frac{X\mu M \times \text{Total volume used for test}}{\text{Time of Incubation}} \times 10^3$$

Determination of Specific enzyme activity :

Specific activity (enzyme activity divided by the amount of protein)

Result:

Conclusion:

2.1 Immobilization of Amylase using calcium alginate Introduction:

Immobilization is a technique for the attachment of a biocatalyst with an insoluble support matrix. The matrix is usually of high molecular weight polymer such as polyacrylamide, starch, cellulose, sodium alginate etc. The advantage of immobilizing enzymes or cells over free cells is to increase their stability and efficiency. The immobilized enzymes or cells can also be recovered at the end of the reaction and can be used repeatedly.

There are five methods for the immobilization of enzymes or cells.

1.**Adsorption:** It is a method which involves electrostatic interaction such

as Van der Waals forces, ionic and hydrogen bonding between the enzymes or cells and the support matrix.

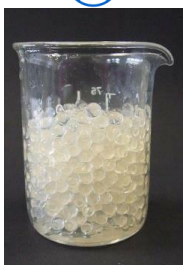
2. **Covalent Binding:** This method involves the formation of covalent bonds between the enzymes or cells and the support matrix. The bond is normally formed between the functional groups present on the 4 surfaces of the support and functional groups belonging to amino acid residues on the surface of the enzyme.
3. **Entrapment:** In this method, the enzyme molecules are mixed with a poly ionic polymer material and then crosslinking of the polymer with multivalent cations is carried out in an ion exchange reaction to form a lattice structure that traps the enzymes or cells.
4. **Encapsulation:** This can be achieved by enveloping the enzymes or cells within various forms of semipermeable membranes.
5. **Crosslinking**: This involves the joining of enzymes or cells with each other to form a large three-dimensional complex structure and can be achieved by physical or chemical methods without any support system

Requirement:

1. 0.1 M phosphate buffer (pH 7)
2. 4% sodium alginate
3. 0.3M calcium chloride solution

Protocol: Enzyme Immobilization

1. Add 4% solution of sodium alginate in 0.1 M phosphate (sodium) buffer (pH 7) by warming at 50^0C.
2. Allow it to cool at room temperature, and then add 1milliliter of enzyme stock solution earlier mixed with 9 milliliter of sodium alginate solution. Mix well.
3. Suspend the mixture dropwise into a pre-chilled 0.3M calcium chloride solution. Gently stir the mixture at 4°C for 2 hrs.
4. Recover beads formed by filtration and wash with distilled water.
5. Store beads in 0.1 M phosphate (sodium) buffer (pH 7.0) at 4°C.
6. Perform enzyme assay by using 1% starch as substrate and estimate the concentration of maltose by DNSA method using standard graph
7. Calculate enzyme activity and specific activity of the immobilized enzyme to compare it with the free enzyme.



Observation:

Calculation:

Results:

Conclusion:

Uses of enzyme amylase:

1. Amylases are used commercially for the preparation of de-sizing agents and removal of starch sizing from woven cloth.
2. Liquefaction of heavy starch pastes formed during heating steps in the manufacture of corn and chocolate syrups.
3. Preparation of sizing pastes for paper coating.
4. Production of bread and removal of food spots in the dry-cleaning industry as in association with proteases, the amylase gets activated.
5. In the brewing industries amylases also can be used as areplacement for maltose for starch hydrolysis.

Practical 3

ISOLATION AND ENUMERATION OF BACTERIOPHAGE

Introduction:

Bacteriophages may be isolated from many natural environments; they are sought where their host cells are found. Under normal conditions, the number of phages in the ecosystem is not high and hence enrichment is the first step toward its isolation. The presence of phage is then demonstrated by the agar overlay method.

Requirements:

1.Phage lysate.
2.Sterile Luria broth in the flask.
3.Chloroform / Membrane filter assembly.
4.Sterile Nutrient broth.
5.Sterile Petri-plates.
6.Sterile Luria agar butts.
7.Sterile nutrient agar plates
8.E. coli-24 hr. old culture.

Procedure:

I) Enrichment:

1.Inoculate the host culture E. coli in nutrient broth and incubate overnight at 37°C to get a turbid culture.
2.To this culture add lysate and incubate overnight.
3.Check reduction in turbidity.

II)Test to detect the presence of phage:

1.Spot inoculate the lysate on nutrient agar plates, which are already seeded with host E.coli cells.
2.Incubate the plates at 37°C for 24 hrs.
3.Observe the development of a clear zone at the site of the spot which indicates the presence of phage.

III)Enumeration by Agar Overlay Method:

1. Inoculate the host culture in broth and incubate at 37°C for 18 hrs. Use this host for the experiment.
2. Use the above host suspension to inoculate fresh luria broth and incubate at 37°C for 2 hrs.
3. Serially dilute the lysate using a sterile phosphate buffer of pH 7.0.Select a suitable dilution to get well-isolated plaques.
4. Add 0.1 milliliter of the diluted lysate +0.1 milliliter of sterile 20%maltose solution+0.1 milliliter of Calcium- Magnesium buffer and 0.2 milliliter of freshly inoculated host and keep at 370C for 10 min. for adsorption. Prepare serial dilutions.
5. Mix this above mixture with 2.5milliliter molten soft agar and pour on the surface of Luria agar plates.
6. Allow to solidify & incubate the plates at 37°C for 24 hrs.
7. Count the number of plaques and calculate PFU/milliliter.

Observation:

Calculation:

Result:

Conclusion:

Practical: 4

Isolation of plasmid DNA & Agarose gel electrophoresis

Introduction:

Plasmids are self-replicating, extrachromosomal, double-stranded, circular DNA molecules Plasmids are replicated by the cell's DNA replication enzymes and inherited by progeny bacteria. They typically contain a few thousand base pairs of DNA and encode few proteins, Often contain genes for drug resistance or for poisons to kill rival bacteria. Plasmids are the favorite tool of biotechnologists because they are small and convenient to work. They are convenient to work. They are the principal tools for inserting new genetic information into microorganisms or plants.

There are several methods to purify plasmid DNA from bacteria These methods invariably involve three steps.

1.Growth of the bacteria.

2.Harvesting and lysis of the bacteria.

3.Purification of plasmid DNA.

Name of the method: Alkaline lysis Meyhod.

Principal:

In this alkaline lysis method cells are lysed by the addition of NaOH and sodium dodecyl

Sulphate (SDS), which denatures plasmid as well as chromosomal DNA. When the solution is Neutralized plasmid DNA gets renatured and remains in the solution along with RNA, while it causes proteins and high molecular weight DNA (Chromosomal DNA) to precipitate, which is separated by centrifugation. Plasmid DNA is then precipitated from the solution by ethanol or isopropanol.

Solution I contain glucose- to prevent immediate lysis of the spheroplast, Tristo maintain pH and EDTA-to chelate the metal ions.

Solution II contains NaOHto denature chromosomal and plasmid DNA and SDSto denature bacterial proteins and destroy the lipid membrane.
Solution III contains potassium acetate to renature the plasmid DNA.
Solution IV contains isopropanol to precipitate DNA.

Protocol:

Day 1:

Under aseptic conditions take one or two loopful of bacterial stock and streak on to LB agar plate containing 100µg/milliliter ampicillin to get isolated colonies. Incubate the plate overnight at 37°C.

Day 2:

Pick a single colony from the plate and inoculate to 10milliliter of LB broth containing 100 µg/milliliter ampicillin. Incubate the tube overnight at 37 °C in the shaker.

Day3:

1. Place 1.5milliliter of the culture in a 1.5milliliter microcentrifuge tube. Centrifuge at 10,000 rpm for 5 minutes.
2. Discard the supernatant and drain the liquid by inverting it on the blotting paper. Keep the tube on ice.
3. Resuspend the cell pellet in 100µl of ice-cold solution to get uniform suspension by vortexing gently. Keep the vial at room temperature (RT) for 5 minutes
4. Add 200 µl of solution II and gently mix the contents by inverting the tube five times. (DO NOT VORTEX). Solution II addition should be done at RT. For 5minutes.
5. Add 150 µl of solution III and gently mix the contents by inverting the tube. Place the iceon for 5 min
6. Centrifuge at 10,000 rpm for 10 minutes
7. Transfer the supernatant to a fresh tube and add 450 µl of solution IV. Mix the contents by inverting the tube and keep the tube at RT for 5-10 minutes for complete precipitation of DNA
8. Centrifuge at 10,000rpm for 10 minutes. Note the orientation of the tube and the expected position of the pellet. Remove the supernatant very carefully, by sliding the pipette tip down the wall of the tube opposite to the pellet and sliding the pipette tip down the wall of tube opposite to the pellet and slowly withdrawing the supernatant. Discard the Supernatant. DNA will be seen as white precipitate sticking to the wall of the tube
9. Wash the pellet with 1milliliter of 70% ethanol. To wash the pellet, add

1milliliter of 70% ethanol without disturbing the pellet and mix by inverting the tube 5 to 10 times

10. Centrifuge at 10,000rpm for 10 minutes. Remove and discard the supernatant in step 8. Dry the tube completely by keeping the cap of the tube open for 10-15 Minutes
11. Add 20 µl of TE buffer and gently tap the sides of the tube to dissolve the DNA. Let it stand at RT with intermittent mixing for 20-30 minutes
12. Add 5 µl of RAase A and incubate at 37 °C for 30 minutes or 42 °C for 5 minutes
13. Check the Plasmid DNA by 0.8% agarose gel electrophoresis. Load 20 µl of plasmid DNA in well.

Electrophoresis:

1.Prepare 1X TAE by diluting the required amount of 50X TAE buffer in 50 with Deionized or distilled water.
2.Take 40milliliter 1X TAE in a 250milliliter conical flask. Add 0.32gm agarose. Boil the mixture to dissolve agarose until the clear solution of agarose is formed. Swirl the mixture to mix and let it cool to 60 °C for safe handling.
3. Meantime seal the ends of the gel tray with tape.Place the comb at one end of the tray, making sure it does not touch the bottom of the tray but is close to it.
4. Pour enough agarose, slowly into the gel tray to cover the lower third of the Comb.Care should be taken not to create any air bubbles. Allow the agarose to cool (it will become whitish and opaque).
5. Remove the tape from the gel tray without damaging the ends of the gel. Place the gel tray into the electrophoresis chamber with the comb nearest the negative electrode end (black leads) of the chamber.
6. Fill the electrophoresis chamber with 1X TAE buffer. The buffer must completely cover the gel.

Staning (By Visual Dye):

1. Carefully remove the gel with the help of a gel detacher from the electrophoresis chamber and place it in the staining box.
2.Dilute 10X DNA staining dye with distilled or deionized water and add this dye solution to the box just to cover the gel (For 100milliliter, take 10milliliter of 10X dye and 90milliliter of distilled or deionized water). Soak the gel in the stain for one hour by shaking every 10 minutes or using the rocker to mix. Remove as much as possible of the staining dye from the staining tray and store it in the container (this dye can be reused).

3. Rinse the gel carefully with tap water or deionized water
4. Cover the gel with fresh tap water or deionized water and soak it for 3 to 5 minutes. Pour off the water.
5. Repeat step 4 until you can see the darkly stained DNA bands against a light blue.

Observation:

Figure: Electrophoresis assembly

Result:

Conclusion:

Partical no 5:

Study of Mitotic cell division from onion root tips

Introduction:

In mitosis, the nucleus of the Eukaryotic cells divides into two, subsequently resulting in the splitting of the parent cells into two daughter cells. Hence, every cell division involves two chief stages.

- Cytokinesis – Cytoplasm division
- Karyokinesis – Nucleus division

Stages of Mitosis

The various stages of mitosis are:

1.Prophase

- The process of mitosis is initiated at this stage wherein coiling and thickening of the chromosomes occurs
- Shrinking and hence the disappearance of the nucleolus and nuclear membrane takes place
- The stage reaches its final state when a cluster of fibres organizes to form the spindle fibres

2.Metaphase

- Chromosomes turn thick in this phase. The two chromatids from each of the chromosomes appear distinct
- Each of the chromosomes is fastened to the spindle fibres located on its controller

Chromosomes align at the centreline of the cell

3.Anaphase

- Each of the chromatid pair detaches from the centromere and approaches the other end of the cell through the spindle fibre
- At this stage, compressing of the cell membrane at the centre takes place

4.Telophase

Chromatids have reached the other end of the cell

The disappearance of the spindles

- Chromatin fibres are formed as a result of the uncoiling of daughter chromosomes
- The appearance of two daughter nuclei at the opposing ends due to the reformation of the nucleolus and nuclear membrane.
- At this phase, splitting of the cell or cytokinesis may also occur.

Post mitosis, the next stage is referred to as interphase, which is part of the cell cycle that is non-dividing and between two consecutivecell divisions. A cell spends most of its life in the interphase. It comprises the G1, S and G2 stages.

Procedure:

1. Take an onion and place it on the title.
2. Carefully remove the dry roots present using a sharp blade.
3. Grow root tips by placing the bulbs in a beaker filled with water.
4. New roots may take 3–6 days to grow.
5. Cut off 2–3 cm of freshly grown roots and let them drop into a watch glass.
6. Using forceps, transfer them to the vial containing freshly prepared fixative of aceto- alcohol (1:3: glacial acetic acid: ethanol).
7. Keep the root tips in the fixative for 24 hours.
8. Using a forceps, take one root and place it on a clean glass slide.
9. Using a dropper, place one drop of N/10 HCl on the root tip followed by 2–3 drops of acetocarmine stain.
10. Warm it slightly on the burner. Care should be taken so that the stain is not dried up.
11. Carefully blot the excess stain using filter paper.
12. Using a blade, cut the comparatively more stained tip portion of the root, retain it on the slide and discard the remaining portion.
13. After that, put one drop of water on the root tip.
14. Mount a cover and slip on it using a needle.

15. Now, slowly tap the coverslip using the blunt end of a needle so that the meristematic tissue of the root tip below the coverslip is properly squashed and spread as a thin layer of cells.
16. This preparation of onion root tip cells is now ready for the study of mitosis.
17. Place the slide under the compound microscope and observe the different stages of mitosis.
18. Various stages of mitosis are prophase, metaphase, anaphase and telophase.

Observation:

Result:

Conclusion:

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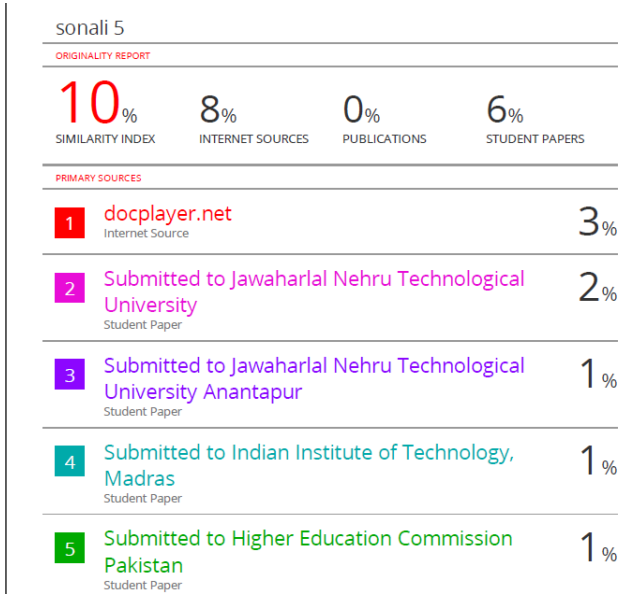

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