ADARSH COLLEGE

OMERGA, DIST. OSMANABAD

DEPARTMENT OF MICROBIOLOGY

CERTIFICATE

Practical paper – III

Certified that Shri / Miss_____

has satisfactorily completed the course of practical work in B. Sc. I YEAR (SEMESTER I & II) prescribed by Dr. B. A. M. University, Aurangabad under my supervision in the Microbiology Laboratory during the academic year 20 – 20

Staff member incharge **Head of the department**

Date: Date:

Signature of the Examiner

Date:

Exam Seat No. _____

Paper III Index				
Sr. No.	**Name of the experiment**	**Date**	**Page no.**	**Signature**
1	Microscopy (Study of Compound Microscope)			
2	Construction, Operation and utility of laboratory equipments			
i)	Autoclave			
ii)	Hot air oven			
iii)	Incubator			
iv)	High speed centrifuge			
v)	Colorimeter			
vi)	Spectrophotometer			
vii)	Laminar air flow			
3	Demonstration of presence of bacteria from – soil/ water/ air/ milk			
4	Demonstration of yeast, fungi, actinomycetes, algae & protozoa.			
i)	Algae			
ii)	Actinomycetes			
iii)	Fungi			
iv)	Yeast			
v)	Protozoa			
5	Microscopic Examination of Bacteria			
i)	Monochrome Staining (Simple Staining)			
ii)	Negative staining (Indirect Staining, Background Staining, Relief Staining)			

iii)	Gram Staining			
6	Hanging drop technique			
7	Micrometry			
8	Qualitative test for			
i)	Carbohydrates (Benedict’s test)			
ii)	Protein (Biuret test)			
iii)	DNA (Diphenylamine test)			
iv)	RNA (Orcinol test)			

Expt. No. 01 Date:

Study of Compound Microscope

Aim: -

To study the different parts of compound microscope.

Theory /Approach: -

Microbiology is the science that deals with the study of all kinds of microorganisms. These are very small and cannot be seen by naked eye. For the observation of microorganisms an instrument Microscope is used. It magnifies the images of microorganisms and makes visible them.

The compound microscope has two parts –

A) Mechanical Parts
B) Optical Parts

Mechanical Parts

1. Foot: - It is heavy and horseshoe shaped. It gives stability to the microscope.

2. Limb: - It is attached to the foot by joint called Hinge Joint. Due to this joint microscope can be tilted at comfortable angle.

3. Draw Tube: - It is attached to the upper end of the limb. External tube and internal tube are the two parts of this tube.

4. Revolving nosepiece: - It is f circular piece, which is attached to the lower end of the external tube. To the revolving nosepiece, interchangeable objective lenses of various magnifications are fitted.

5. Coarse adjustment screw: - With this screw the tube can be adjusted fast in such f way that objective lens can be positioned at its optimum working distance.

6. Fine adjustment screw: - With this, we can do fine adjustment of the tube so that the clear image can be observed.

7. Stage: - It is the square metal plate fitted below the objective lenses on which slide is kept. It has a hole at the center for passing the light from condenser to the object.

8. Rack and Pinion mechanism: - On the stage this mechanism is fitted, with the help of which we can move the slide to and fro and side to side.

B) Optical Parts

1. Mirror: - It is fitted near the foot below the stage. It is plane on one side and concave on the other side. It is used to direct the light from the light source towards the condenser. When the light intensity is low, concave side of the mirror is used.

2. Condenser: - It is fitted below the stage and above the mirror. The glass of condenser collects light from mirror and focuses on the object. The condenser can be raised or lowered with the help of screw thus the intensity of the light is adjusted.

3. Iris diaphragm:- It is placed below the condenser to correct spherical aberrations (excess of light can be adjusted).

4. Objective lenses: - There are three types of objective lenses.

a) Low power lens
b) High power lens
c) Oil immersion lens

Table:

Sr. No.	Objective Lens	Magnification	Focal length	Diameter of field seen	Total magnification with 10X eye piece
1.	Low power	10X	16 mm	1.55 mm	100
2.	High power	40X	04 mm	0. 31 mm	400
3.	Oil immersion	100X	02 mm	0.16 mm	1000

5. Eye piece: - It is fitted at the upper end of the drawtube. Its function is to magnify further the image produced by objective lens and focus it into the eye. Its magnifying power is 10X or 15X.

CARE FOR USING MICROSCOPE

The microscope is most important instrument in microbiology; it should be always used with care and maintained properly. It should be always protected from dust and kept in dean container. It should be always protected from dust and kept in clean Container. It should be cleared at interval. The immersion lens is cleared with Xylene after use to remove oil.

Comfortable for observation check that the objective, eye piece, stage and mirror is totally free from dust.

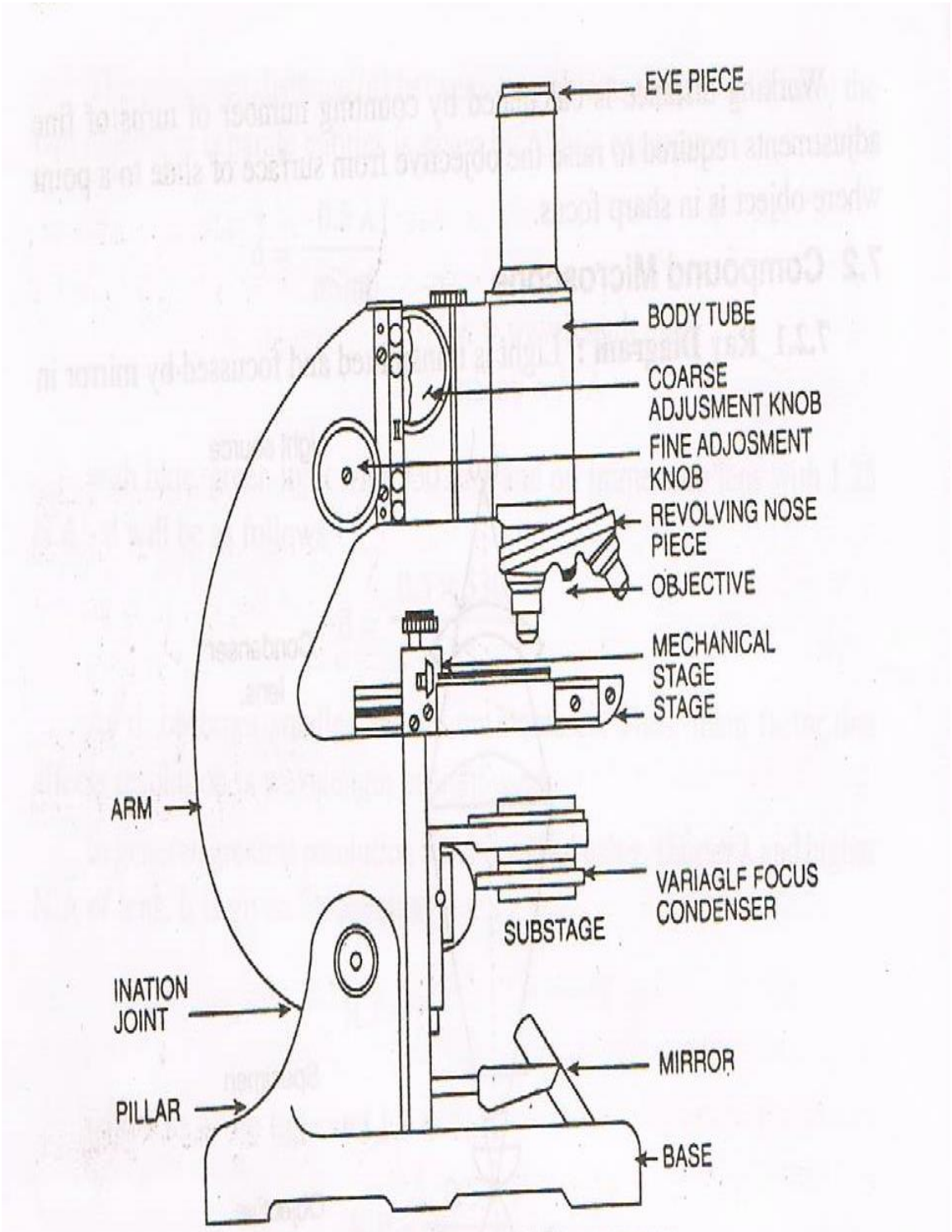


Fig. 7.10 : Compound Microscope

Expt. No. 2 Date:

Construction, Operation and utility of laboratory equipments

i) Autoclave

Construction: -

It is a cylindrical vessel made up of aluminium metal. The capacity ranges from 5 to 100 liter. The lid on its upper side consists of steam valve pressure indicator & safety valve. This lid can be fitted to the vessel by screws on the upper end of vessel. Heating coils are placed at the bottom of vessel, which are heated electrically.

In water, platform is placed & on this pot is placed. In this pot the material to be sterilized is kept.

Principle: -

The boiling point of water is directly proportional to pressure, when volume is constant. Water boils when its vapour pressure is equal to atmospheric pressure. Normally water boils at 100^{0} C temperature but when water is filled within closed vessel by increasing pressure the temperature at which the water boils will increase above 100^{0} C. For sterilization of liquid material, we require 121^{0} C temperature. Due to which all organisms including heat resistant spores are killed. Thus in autoclave 121^{0} C temperature can be created by pressure of steam at 15 lbs / inch ^{2} pressure.

Working: - The material to be sterilized is plugged with nonabsorbent cotton and then covered with paper to avoid removal of plug after keeping the material inside on the platform. The lid is kept in position and screws are fitted. Electric current is supplied to heat water. Due to boiling of water, air present inside the autoclave will come out and is completely removed by water vapour. Then vessel is closed. If air remains inside, the temperature required at particular pressure will not be maintained.

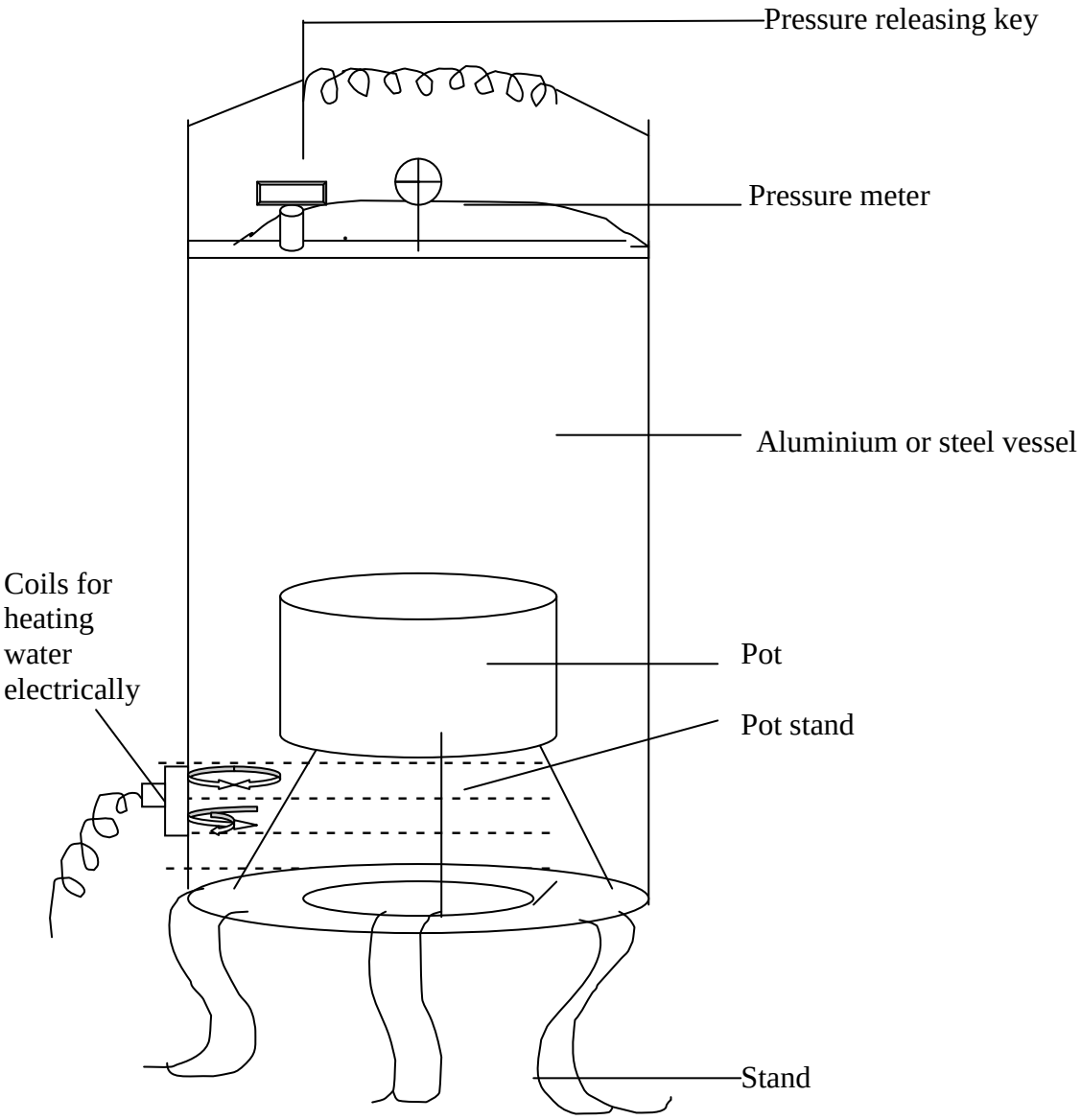
Holding period: - Exact duration of holding period is found according to the nature of material to be sterilized. Usually 15 lbs / inch ^{2} pressure for 15 minutes of time is required for complete sterilization. But for sterilization of sugar medium, 10 lbs / inch ^{2} pressure for 40 minutes is required because at higher pressure sugars are destroyed.

After particular pressure and time, the autoclave is switched off and allowed to cool. After pressure is decreased to 0 lbs / inch^{2} lid is removed and material is taken out.

Precaution: - The pressure must be allowed to decrease slowly after sterilization otherwise fluid in the container will boil over and come out of container.

Mode of action: - Steam hydrates (addition of water) microorganisms and spores thus promote coagulation and denaturation of proteins in microorganisms. Thus death of microorganisms takes place.

AUTOCLAVE


Pressure releasing key
Pressure meter
Aluminium or steel vessel
Coils for heating water electrically
Pot
Pot stand
Stand

Expt. No. 02 Date:

ii) Hot air oven

Construction:-

It is a double walled metal structure insulated with aluminum or asbestos sheet .The capacity of the chamber is divided to compartments by removable racks. There are different sizes of oven .It is heated electrically heating coils situated at the bottom of oven. Above the oven thermometer is inserted to note the temperature. Adjustable exhaust of the air are present to the sidewalls of the oven .The heating of the coil is adjustable with the knob. After adjustment of particular temperature, automatically temperature is maintained.

Holding time: -

In oven dry articles are sterilized. 165^0 C temperatures for two hours is required for sterilization of glass material but more temperature and time is required for metals.

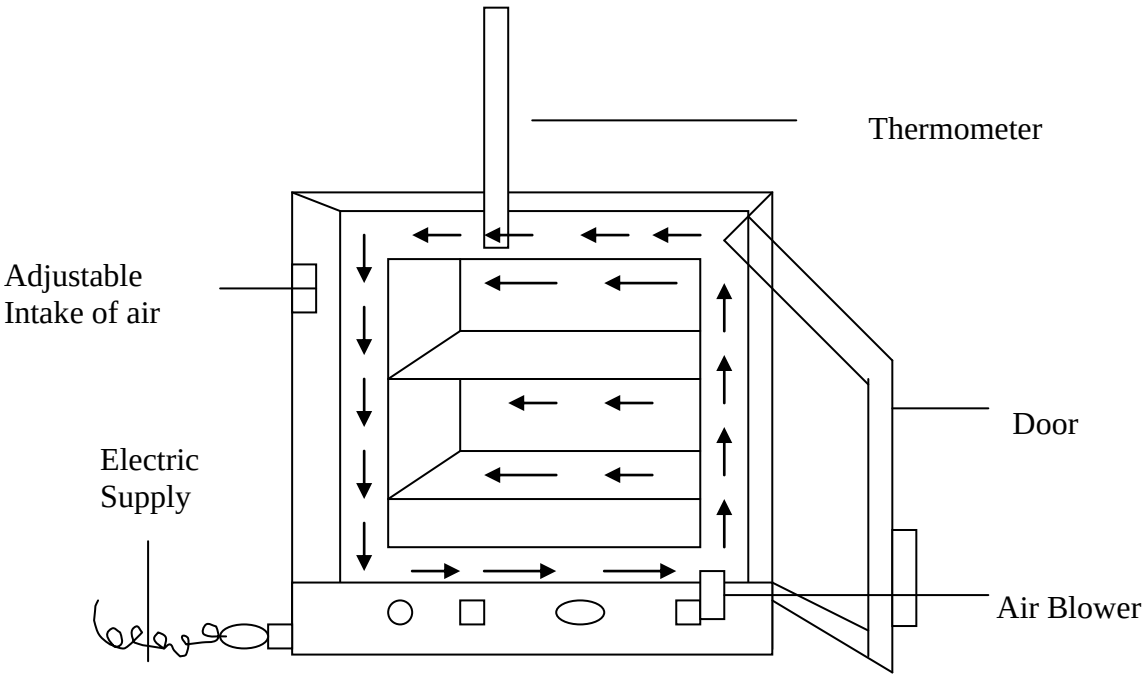
Precaution: -

Glass material should be dried before sterilization. Glass material must be plugged and covered properly with paper. After sterilization electricity is switched off and oven is allowed to cool slowly and then only, the door of the oven is opened. If door is opened at higher temperature, the glass material will be cracked or broken due to sudden decrease in temperature.

Principle: -

Dry heat means dry air with high temperature but without steam and moisture. Sterilization by dry heat is done by hot air oven. Here all organisms and heat resistant spores are killed due to dehydration of cellular material of organisms.

Hot Air Oven



Expt. No. 02 Date:

iii) Incubator

Construction: -

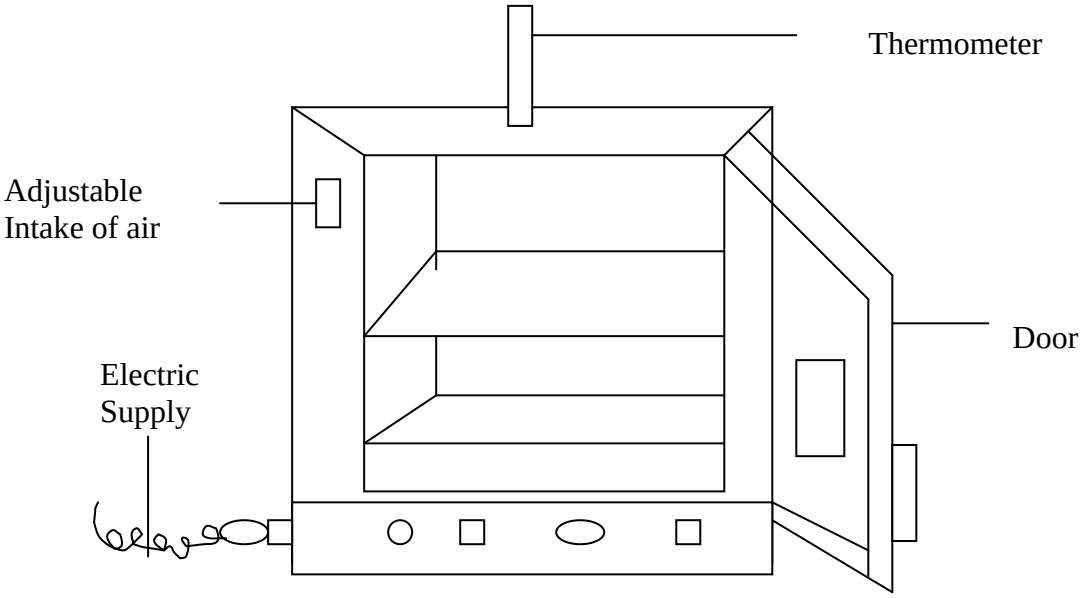
It is a double walled metal structure or compartment insulated with asbestos, made up of aluminum or steel. There are different compartments made up of removable racks. The front part of the chamber is covered with glass; the material to be incubated is kept on the racks. Chamber is heated electrically by heating coils, which are situated at the bottom.

Working: -

Make the incubator on by supplying electrical current; with the help of regulatory knob adjust it at full position. When required temperature is achieved, turn the knob till red light of adjusting knob is just off. Keep this knob in this position for required period. The temperature will be maintained automatically. There is thermometer at the top for observation of temperature. There is no cooling system to lower down the temperature.

Function: -

Incubator is an apparatus used for incubation of microorganisms at their optimum constant temperature and humidity.



Expt. 2 Date:

iv) High speed centrifuge:

- These centrifuges are available with maximum rotor speed of 25000 rpm generating a relative centrifugal field of about 60000 g with a range of interchangeable fixed angle and swinging bucket rotor are available.
- These are recommended to use for collecting microbes, cellular organelles and precipitated proteins.
- Due to low centrifugal force, these can not be used to sediment viruses, and smaller organelle such as ribosome.

  The type of centrifuge is called continuous flow centrifuge. In this type rotor flow is medium but continuous and rotor is long, tubular and interchangeable. These are used for sedimentation of bacteria, yeast cells etc from large volume of culture medium.

Expt. No. 2 Date: -

v) Colorimeter

A **colorimeter** is a device used in colorimetry. In scientific fields the word generally refers to the device that measures the absorbance of particular wavelengths of light by a specific solution. This device is most commonly used to determine the concentration of a known solute in a given solution by the application of the Beer-Lambert law, which states that the concentration of a solute is proportional to the absorbance.

The essential parts of a colorimeter are:

- a **light source** (often an ordinary low-voltage filament lamp)
- an adjustable aperture
- a set of colored filters
- a cuvette to hold the working solution
- a detector (usually a photoresistor) to measure the transmitted light
- a meter to display the output from the detector

In addition, there may be:

- a voltage regulator, to protect the instrument from fluctuations in mains voltage.
- a second light path, cuvette and detector. This enables comparison between the working solution and a "blank", consisting of pure solvent, to improve accuracy.

Filters

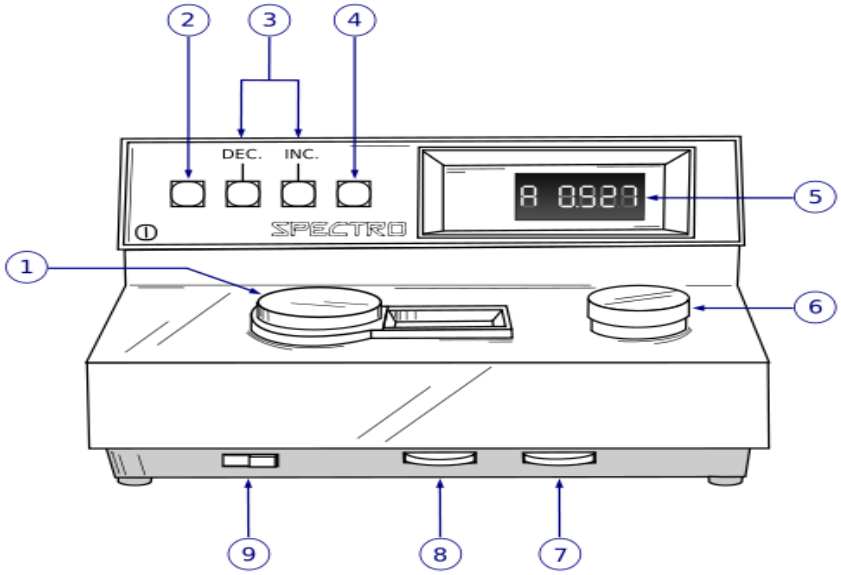
Changeable optics filters are used in the colorimeter to select the wavelength of light which the solute absorbs the most, in order to maximize accuracy. The usual wavelength range is from 400 to 700 nanometers (nm). If it is necessary to operate in the ultraviolet range (below 400 nm) then some modifications to the colorimeter are needed. In modern colorimeters the filament lamp and filters may be replaced by several light-emitting diodes of different colors.

Cuvettes

In a manual colorimeter the cuvettes are inserted and removed by hand.

Output

The output from a colorimeter may be displayed by an analogue or digital meter and may be shown as transmittance (a linear scale from 0-100%) or as absorbance (a logarithmic scale from zero to infinity).



1-Wavelength selection;
2-Printer button;
3-Concentration factor adjustement;
4-UV mode selector (Deuterium lamp);
5-Readout;
6-Sample compartement;
7-Zero control (100% T);
8-Sensitivity switch;

9-ON-OFF switch.

Expt. No. 2 Date: -

vi) Spectrophotometer

Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength.

Spectrophotometry involves the use of a spectrophotometer. A spectrophotometer is a photometer (a device for measuring light intensity) that can measure intensity as a function of the light source wavelength. Important features of spectrophotometers are spectral bandwidth and linear range of absorption or reflectance measurement.

A spectrophotometer is commonly used for the measurement of transmittance or reflectance of solutions, transparent or opaque solids

Ultimately, a spectrophotometer is able to determine, depending on the control or calibration, what substances are present in a target and exactly how much through calculations of observed wavelengths.

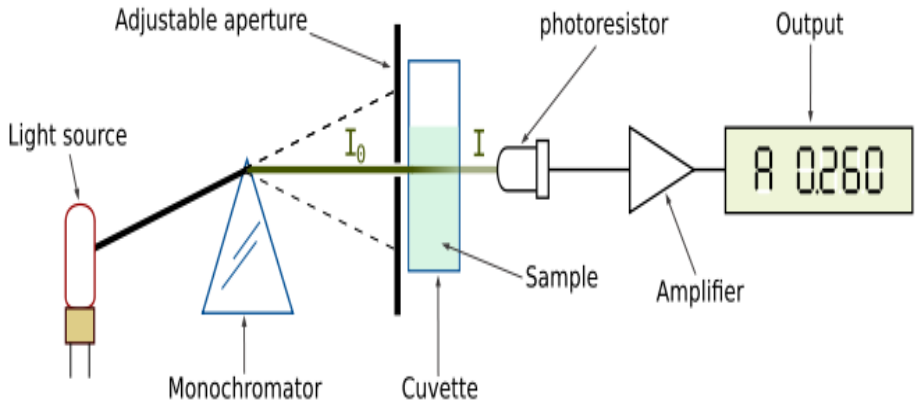
There are two major classes of devices: single beam and double beam. A single beam spectrophotometer measures the relative light intensity of the beam before and after a test sample is inserted. A double beam spectrophotometer compares the light intensity between two light paths, one path containing a reference sample and the other the test sample

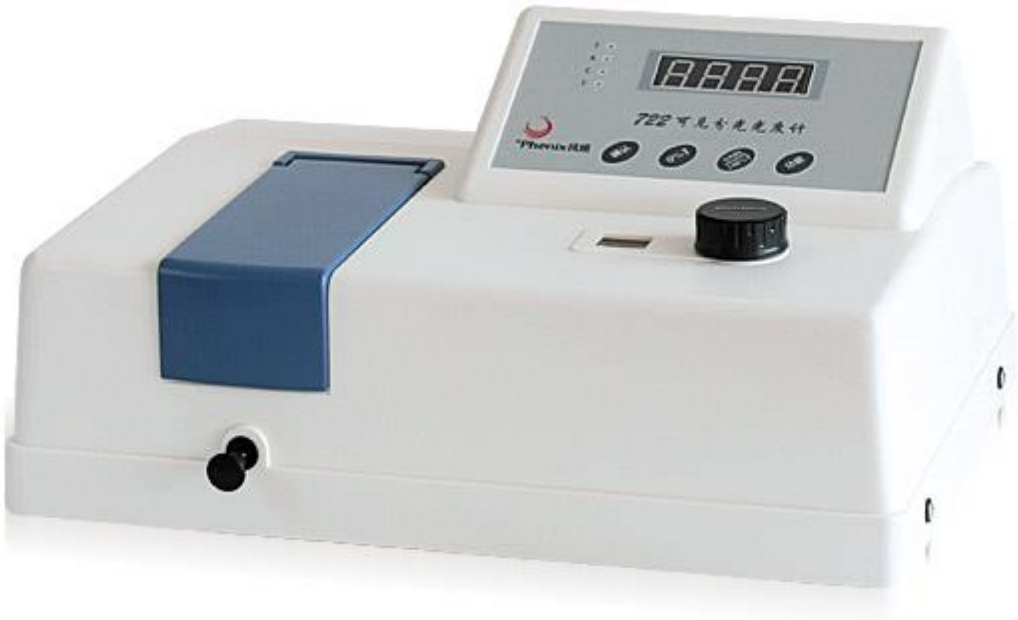
Light from the source lamp is passed through a monochromator, which diffracts the light into a "rainbow" of wavelengths and outputs narrow bandwidths of this diffracted spectrum. Discrete frequencies are transmitted through the test sample. Then the photon flux density (watts per metre squared usually) of the transmitted or reflected light is measured with a photodiode, charge coupled device or other light sensor. The transmittance or reflectance value for each wavelength of the test sample is then compared with the transmission (or reflectance) values from the reference sample.

In short, the sequence of events in a modern spectrophotometer is as follows:

1. The light source is imaged upon the sample
2. A fraction of the light is transmitted or reflected from the sample
3. The light from the sample is imaged upon the entrance slit of the monochromator
4. The monochromator separates the wavelengths of light and focuses each of them onto the photodetector sequentially

Many older spectrophotometers must be calibrated by a procedure known as "zeroing." The absorbancy of a reference substance is set as a baseline value, so the absorbancies of all other substances are recorded relative to the initial "zeroed" substance. The spectrophotometer then displays % absorbancy (the amount of light absorbed relative to the initial substance).[


Adjustable aperture
photoresistor
Output
Light source
I_0
I
A 0.260
Sample
Amplifier
Monochromator
Cuvette



Expt. 2 Date

vii) Laminar Air Flow

The environmental air is drawn by centri-balanced, centrifugal succession blower through pre-filters and is thrown to work plate form through high efficiency H.E.P.A. filters, retaining particulates and biological contaminants larger than 0.3 microns, Table-top is covered with stainless steel sheet.

• All the compartments of the chamber are chemically sealed to prevent leakage/loss of air-pressure, Front door is collapsible type & side panels are fixed, made of crystal clear thick plexi-glass or transparent polycarbonate.

• All variants are available with U.V. Tube, Fluorescent Tube light, Horizontal Laminar Flow made of stainless steel top. Front panel made of toughened glass in two pieces one piece fix and the other sliding upwards. Build with U.V. Germicide light and white light and adequate illumination. Hepa filter efficiency rating 99.9% down to 0.3 microns. Motor blower assembly. Clean air laminar airflow cabinets are designed for creation of a bacterial dust free air space. Laminar air flow cabinets are used for work with low-risk substances and materials, when protection of working material from environment is required or work with item requires a sterile working zone. Cabinets are used for equipment of local workplaces in medical, pharmaceutical and other institutes with high requirements for air cleanliness in the working zone.

Used for inoculations, spreading, streaking of microbial cultures to avoid contamination from air. (Aseptic condition)

Expt. No. 3 Date:

Demonstration of bacteria from soil, water, air and milk

Aim: To isolate different types of microorganisms from soil, water and air.

Theory: All regions of the earth that contain living organisms are known collectively as the "BIOSPHERE". This includes Soil, Water and Air.

Soil is the upper layer of most of the earth's surface and varies in depth from inches to over 20 feet. Microorganisms are present in soil because there is a food for them. Soils are excellent cultural media for the growth of many types of organisms. These include Bacteria, Fungi, Algae, Protozoa and Viruses. A spoonful of soil contains billions of microorganisms. The number and kinds of microorganisms found in the soil depend up on the nature of soil, depth, season of the year, state of cultivation, reaction, organic matter, temperature, moisture, aeration etc.

All metabolic reaction of living organisms depends on the presence of water. Microorganisms get into waters from air, soil, sewage, organic wastes, dead plants & animals etc. Thus any type of microorganisms may be found in water. The numbers and kinds of microorganisms in water depend upon food material, Climatic, geographical and biological conditions.

Air is not a natural environment for the growth of microorganisms. It does not contain necessary amount of moisture and nutrients. The microorganisms found in air come from soil, organic wastes of man and animal, the oral, nasal and rectal passages of man and animals and from the lungs through coughs and sneezes. The kinds and numbers of microorganisms in the air vary, depending upon the sources of contamination in the environment and locality and also speed of air current, humidity, sunlight, temperature and the size of particles on which they are attached.

Milk is good culture medium for the growth of microorganisms as it contains proteins, carbohydrates, fats, vitamins and minerals. Microorganisms enter in the milk by different sources such as milking animal and man, utensils, during storage and transportation.

Requirements:

1. Sterile nutrient agar plates
2. Sterile saline tube

Procedure:

A) Isolation of bacteria from soil:

One gram of soil is added in 10 ml sterile saline. A loopful from this is streaked on sterile nutrient agar plate aseptically. The plate is incubated at 37 ^{o} C for 24 hours. After incubation colony characters and Gram nature are studied of well-isolated colonies.

B) Isolation of bacteria from water:

A loopful of water sample is streaked on sterile nutrient agar plate aseptically. The plate is incubated at 37 ^{o} C for 24 hours. After incubation colony characters and Gram nature are studied of well-isolated colonies.

C) Isolation of bacteria from air:

Sterile nutrient agar plate is exposed to air for 5 minutes. The plate is incubated at 37 ^{o} C for 24 hours. After incubation colony characters and Gram nature are studied of well-isolated colonies.

C) Isolation of bacteria from milk:

A loopful of milk sample is streaked on sterile nutrient agar plate aseptically. The plate is incubated at 37 ^{o} C for 24 hours. After incubation colony characters and Gram nature are studied of well-isolated colonies.

Observations: Different types of colonies are observed on nutrient agar plates. Following are the colony characters and Gram nature of selected colony from each plate.

Colony Characters:

Colony characters	Soil	Water	Air	Milk
Size				
Shape				
Colour				
Margin				
Elevation				
Opacity				
Consistency				
Gram nature				

Result:

From observation it can be said that there are different types of bacteria in the soil water and air.

Expt. No. 4 Date:

Demonstration of yeast, fungi, actinomycetes, algae & protozoa.

Study of Algae

Anabaena;

Group: *Cyanobacteria*

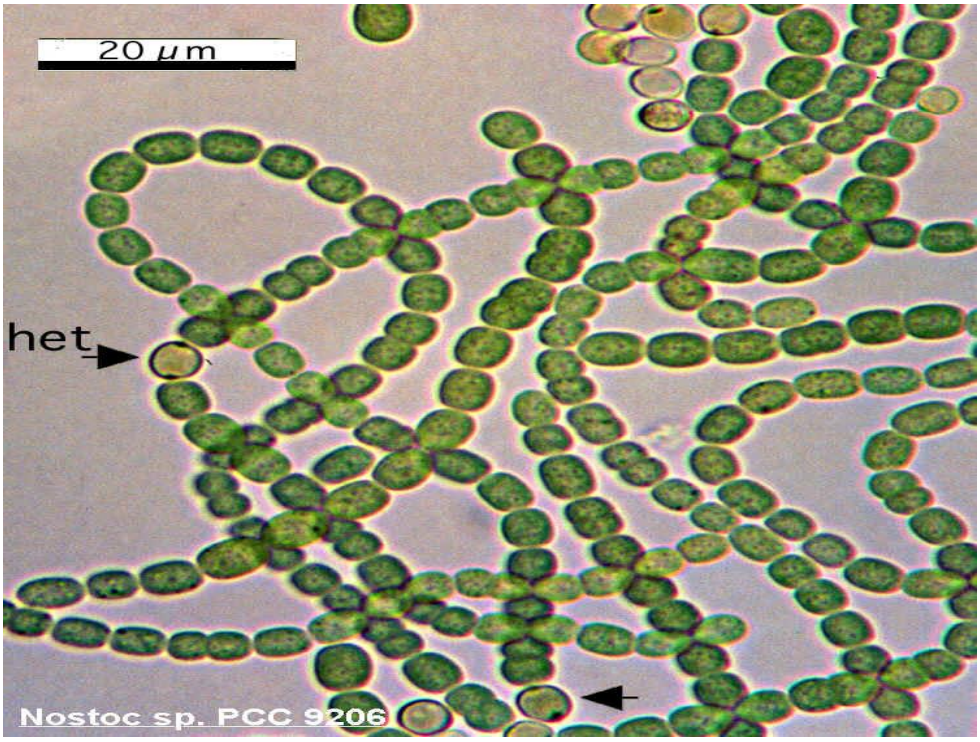
Order: *Nostocales*

Family: *Nostocaceae*

Genus*: anabaena*

- They are oxygenic photoautotrophic bacteria in which akinetes are usually formed.
- They are cylindrical, spherical or ovoid forms generally having 2-10 um in size.
- The plant body consists of vegetative cells as well as heterocyst and akinetes.
- The heterocyst are present either intercalary or in terminal or both position.
- The cells contain slime covering and a distinct individual sheath is absent.
- The trichomes are normally motile and colonies are not formed.
- Their species establish symbiotic association with fungi.
- Examples are; *A.azollae, A.cycadae.*

Nostoc:



General characters: -

1. These are photosynthetic eukaryotes.

Classification: -

Kingdom	*Protista*
Division	Cyanophyta
Class	Cyanophyceae
Order	Nostocales
Family	Nostocaceae
Genus	Nostoc

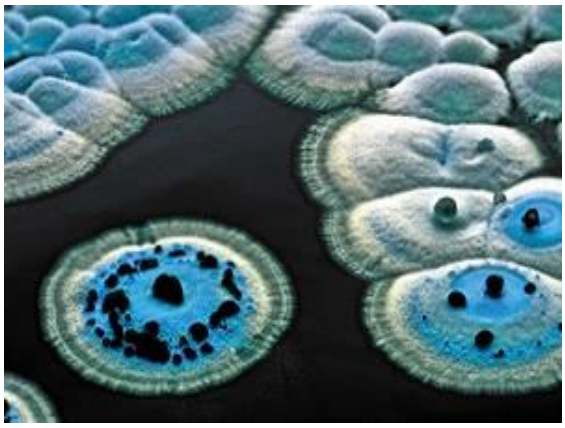
Study of ACTINOMYCETES

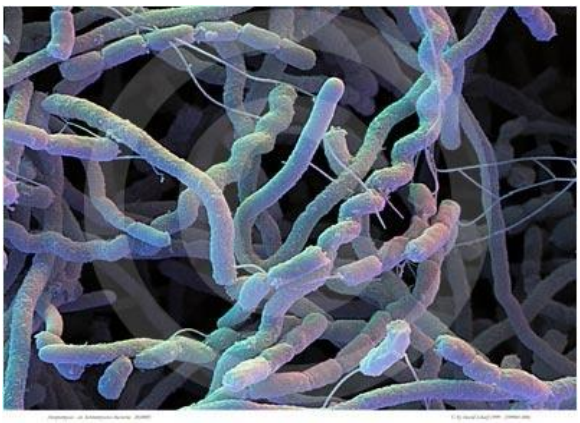
Streptomyces

Section*; streptomycetes* and related genera

Genus; *streptomyces*

- They are filamentous bacteria, consist of vegetative hyphae, produced branched mycelium that rarely filaments.
- Aerial mycelium at maturity, form chains of 3 to many spores which are non motile.
- The colonies are discrete, lichenoid, leathery or butyrous on solid medium and produce a wide variety of pigments responsible for the color of the vegetative and aerial mycelium.
- The colored pigments are diffusible in the medium.
- Mostly, these are Gram positive but not acid alcohol fast.
- They are aerobic and catalase positive.
- They use a wide range of organic compound as a C source.
- The temperature varies from 25-35’ C and pH ranges from 5-8.
- They are abundant in soil.



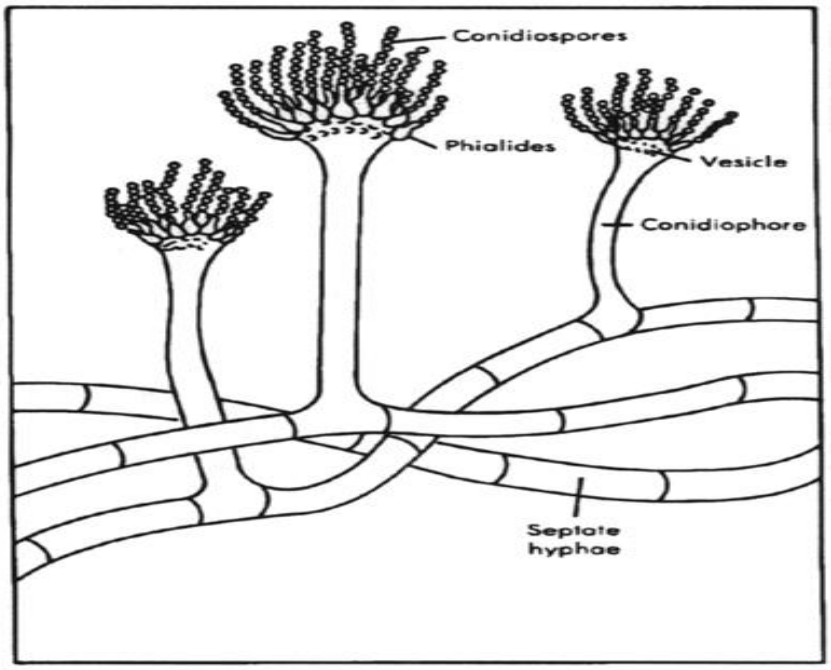


Economic importance

1. Most species produce antibiotics
2. Degrade many components in soil

Study of FUNGI

1. Aspergillus



General characters: -

1. It is saprophytic black coloured mold.
2. They reproduce sexually and asexually. Asexual spores are called as conidia.
3. Conidia are produced on sterigmata.
4. Mycelium is septate.

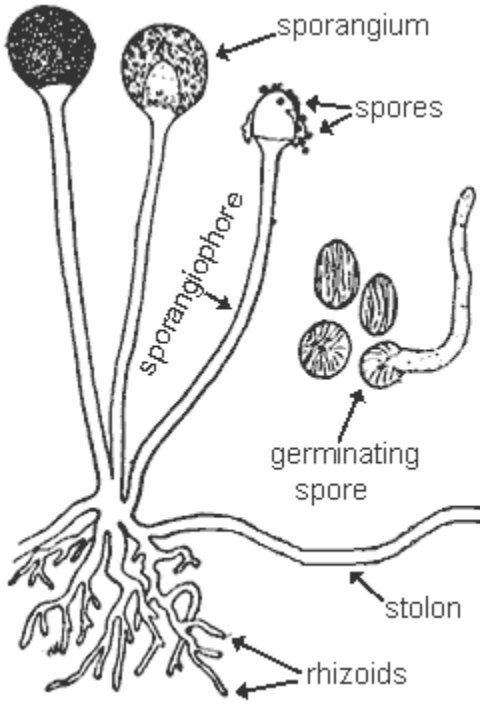
Classification: -

Kingdom	Protista
Division	Mycota
Subdivision	Higher fungi
Class	Ascomycetae
Family	Eurotiacae
Genus	Aspergillus

Economic importance

1. *Aspergillus niger* is used in industries for citric acid production
2. *Aspergillus flavus* produces Afla toxin which is carcinogenic.
3. *Aspergillus fumigatus* causes Aspergillosis disease.
4. *Aspergillus species* cause distruction of food, textile, paper, leather etc.

2. Rhizopus: -



General characters: -

1. It is saprophytic white coloured mold. Also called bread mold.
2. They reproduce sexually and asexually. Asexual spores are called as sporangiospores.
3. Sporangiospores are produced in sporangium.
4. Mycelium is non-septate.

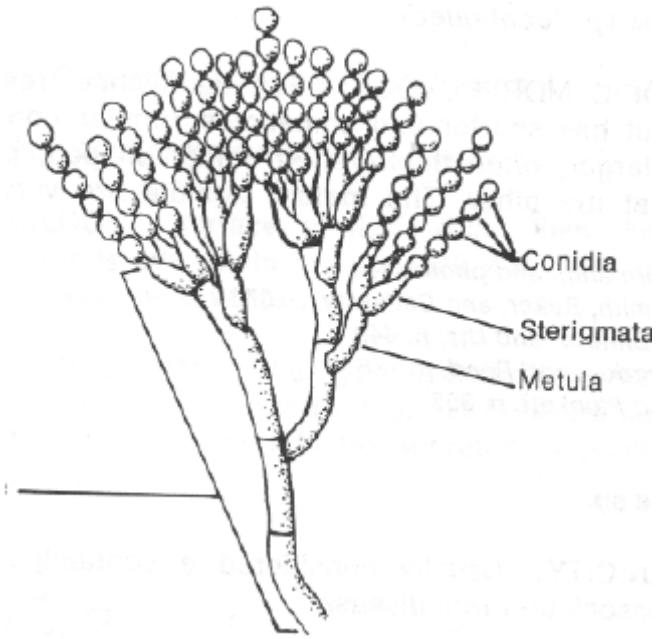
Classification: -

Kingdom	Protista
Division	Mycota
Subdivision	Lower fungi
Class	Zygomycetes
Order	Mucorales
Genus	Rhizopus

Economic importance

1. Involved in food spoilage particularly bread, pickles, jams, jellies and sausages.
2. Many produce plant diseases.

Penicillium:



Class*; Deuteromycetes*

Order; *Moniliales*

Family*; Moniliaceae*

- Colonies are of various colors (green, bluish green) central area raised or smooth, may be zonate with age, radially furrowed.
- Vegetative hyphae creeping, wooly or cottony, floccose to semi floccose, slow or fast growing.
- Hyphae branched and septate producing branched or unbranched conidiophores each with one, two or more verticil of phialides, and metulae,(secondary and some times primary branchlets).
- Conidia borne in chains typically forming brush like head, not enclosed on slime, well differentiated footcells not present.
- Conidia globose, ovate or elliptical with smooth or rough surface.
- The species that produce penicillin's are *P.notatum, P.chrysogenum.*

STUDY OF YEAST:

Saccharomyces cerevisiae

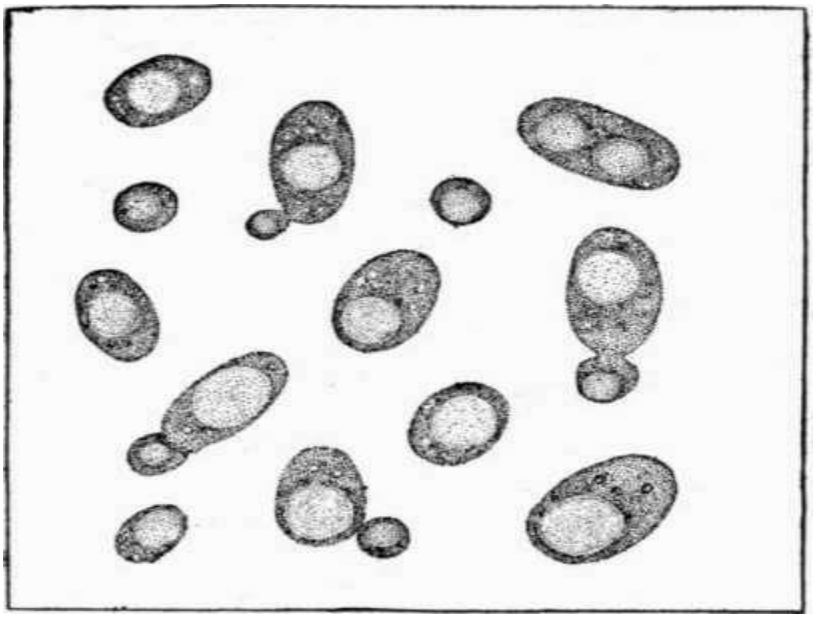
Class; *Ascomycetes*

Order; *Endomycetales*

Family; *Saccharomycetes*

Genus*; saccharomyces.*

- Mostly it is saprophyte and found every where provided there is sugary substrate, like flowers, fruits, soil, animal excreta, milk and fermented milk product.
- It is commonly used in bread making, hence called brewer's or baker's yeast as well as wine yeast and distiller's yeast.
- It is unicellular edible fungus, its thallus is non mycelial.
- It consists of single minute oval or spherical cell. Its size ranges \from 2-8um.
- Cells are hyaline in colour consisting of protoplast covered by a cell wall.
- Asexual reproduction takes place by binary fission and budding, sexual reproduction is commenced through plasmogamy and meiosis.
- The cells secrete enzymes on the substratum which are collectively called zymases. The enzyme change starch or complex sugar into simple sugars.



PROTOZOA

Entamoeba histolytica;

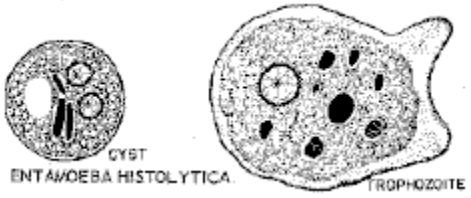
Phylum; P*rotozoa*

Class; *Rhizopoda*

Order*; Lobosa*

Genus*; Entamoeba*

Species*; histolytica*



- The amoeba is parasitic in the intestine and transmission from man to man occurs through cyst.
- The cysts are round, oval, in out line, refractile, pearly in colour having a definite wall. Cyst may contain 1-4 nuclei and 12 mm in diameter.
- The uncysted amoeba consists of a small mass of cytoplasm differentiated into outer ectoplasm and inner dense mass called endoplasm.
- If the amoeba has been isolated from faeces of human being, the ingested red cells appear of small dimensions than the free red cells, which are also present in the red cells.
- The amoeba invades the mucous membrane of large intestine and multiplies in it.

Expt. No. 05 i) Date:

Monochrome Staining
(Simple Staining)

Aim: -

To observe morphology and arrangement of the bacterial cells by monochrome or simple staining method.

Theory /Approach: -

Bacteria are very small in size. They cannot be observed with naked eye. To observe them, an instrument is used called as microscope. Bacteria are colourless; there is no contrast between surrounding material and bacteria. Therefore they are not observed clearly. By staining of bacteria, they can be observed as colored cells.

The chemical compound used to give colour to the bacteria is called as stain or dye. Dye contains both Auxochrome and Chromophore group. The auxochrome group gives the property of electrolytic dissociation to the dye so that dye posses affinity and ability to bind with other material. Chromophore group gives the property of colour to the dye. Depending upon the chromophore group the stain may be basic (possessing positive charge) e.g. Crystal violet, Methylene blue, Saffranin, Basic fuchsin etc. or it may be acidic (possessing negative charge) e.g. Nigrosin, Eosin, Congo red, India ink etc.

Basic stain stains acidic parts and Acidic stain stains basic parts of bacterial cell.

Mechanism of staining: -

The basic stains have strong affinity towards acidic portion of the cell. The surface of the bacterial cell and cytoplasmic components have overall acidic characteristics. The chromophore group of basic stain is positively charged (cation). Therefore it can easily form ionic bonds with the acidic molecules.

If f single dye is used to stain the bacteria, the method is called as simple staining or Monochrome staining.

Requirements: -

1. Suspension of bacterial cells
2. Clean grease free glass slide
3. Glass marking pencil
4. Nichrome wire loop
5. Basic stain solution (Crystal Violet, Methelene Blue, Saffranin)
6. Microscope with 100X oil immersion lens
7. Cedar wood oil

Procedure: -

1.On a clean grease free slide a circle is marked on its lower side with the help of glass marking pencil.

2. With the help of sterile nichrome wire loop, a loopful of suspension is taken in aseptic condition.

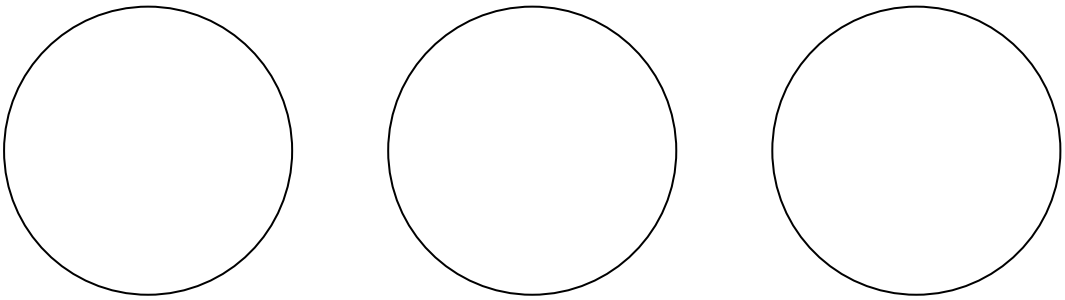
3. A smear is prepared on the slide on the opposite surface of marked area.

4. Smear is air-dried, heat fixed by passing the slide gently through the flame twice or thrice.

5. The smear is flooded with given stain and allowed to react for two minutes. Then slide is washed with tap water gently.

6. Smear is then air dried and observed under oil immersion lens putting a small drop of cedar wood oil on the smear.

Observation: -



1. Violet coloured, _____ shaped bacteria are observed after staining with crystal violet.

2. Blue coloured _____ shaped bacteria are observed in bunch, in pairs and single after staining with methylene blue.

3. Pink coloured _____ shaped bacteria are observed after staining with saffranin.

Result: -

With the help of different stains, shape and arrangement of bacteria is observed.

Conclusion: -

In monochrome staining, only one stain is used. Surface of the bacterial cell is acidic and is negatively charged. Therefore basic stain containing positively charged chromophore group formed ionic bonding. In monochrome staining we can observe shape and arrangement of bacterial cells clearly. This method is very easy and simple.

Expt. No. 05 ii) Date:

Negative staining
(Indirect Staining, Background Staining, Relief Staining)

Aim: -

To observe the morphology of the bacteria by Negative staining method.

Theory: -

Negative staining is a technique by which bacterial cells are not stained but are made visible by staining the background dark. Acidic stains like Nigrosin, Eosin, Congo red, and India ink etc. are used for this method. Acidic stains do not have affinity towards bacterial cells because bacteria are also acidic in nature (negatively charged) hence; there is repulsion between acidic stains and negatively charged bacteria.

The size and shape of the bacteria can be much more accurately determined by this technique but internal structures cannot be observed.

This technique is advantageous over positive staining technique because it is very easy for the study of cell morphology and cell do not receive vigorous physical and chemical treatments.

In this technique heat fixation is avoided because it may disturb the cells or shrinkage of the bacterial cells occurs. This staining is called as background staining because background is stained, relief staining because bacteria get relief from physical and chemical treatments and indirect staining because bacteria are not stained directly.

Mechanism: -

The chromophore group of acidic stain has negative charge. Hence it can not combine with negatively charged bacterial cell. On the other hand, acidic stain forms background of bacterial cells, thus resulting into colourless appearance of bacterial cells against the dark background.

Precaution: -

Thick smear of stain and suspension may result cracking hence very thick smear should be avoided. Very thin smear will not give dark background hence very thin smear should be avoided.

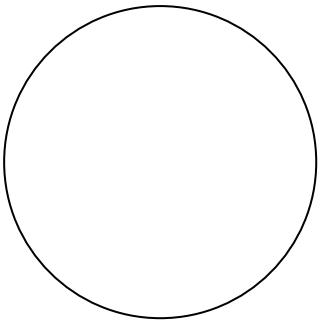
Requirements: -

1. Suspension of bacterial cells
2. 10 % aqueous Nigrosin solution

Procedure: -

1. The drop of suspension and the drop of Nigrosin is taken on a clear grease free slide.
2. These two drops are mixed with nichrome wire loop or with another slide.
3. This drop is then spread with the help of nichrome wire loop or with another slide so as to form thin film.
4. This film is air dried and observed under oil immersion lens.

Observation: -



Colourless _____ shaped bacteria are observed against the black coloured background.

Result: -

With the help of this method the morphology of the bacteria is observed with their original colourless property.

Conclusion: -

In this method acidic stain is used. As the surface of the bacteria is acidic in nature, acidic stain does not stain bacteria but stains background. Hence we can observe colourless bacteria and their morphology and arrangement in their original form.

Expt. No. 05 iii) Date:

Gram Staining

Aim: -

To stain and differentiate the bacteria in the given suspension by Gram staining method.

Theory: -

It is one of the most important and widely used differential staining method in microbiology. The scientist Christian Gram in 1884 of Danish discovered this method. This method differentiates bacteria into gram positive and gram-negative cell types.

Mechanism: -

In this method, the difference in chemical composition of cell wall is used to differentiate bacteria. Here primary stain crystal violet is used. Gram's iodine combines with crystal violet to form an insoluble complex that is Crystal Violet Iodine (CVI) complex.

The decolourization step is done by absolute ethanol and then counter stain saffranin is applied.

Some bacteria appear violet in colour and are called Gram positive while others appear red in colour and are called Gram negative. In gram-positive bacteria CVI complex is not removed by ethanol, therefore they appear violet. In gram-negative bacteria CVI complex is removed by ethanol and they become colourless, they get stained by counter stain saffranin and appear red in colour.

To explain the mechanism of Gram staining or the ability of the bacteria to retain CVI complex or to give up CVI complex or why some bacteria get decolourised and others do not by ethanol, different theories have been put forward. The most accepted theories are ---

1. Lipid content theory
2. Peptidoglycan content theory
3. Magnesium ribonucleate content theory

1. Lipid content theory: -

Lipid is more in Gram-negative bacterial cell wall. When ethanol is applied, lipid is extracted from cell wall and thus pore size and permeability of cell wall increases, due to which CVI complex is removed easily and bacteria are decolourised. When saffranin is applied they take red colour of it and are called as Gram negative.

In Gram-positive bacteria lipid is less in cell wall, when ethanol is applied, dehydration of cell wall takes place, and hence pore size and permeability decreases. Thus CVI complex is not removed and bacteria appear violet in colour. When saffranin is applied, it does not stain already stained bacteria by CVI complex.

2. Peptidoglycan content theory: -

In Gram- positive bacteria, peptidoglycan is more. Ethanol treatment causes decrease in pore size of cell wall and CVI complex is trapped and bacteria appear violet in colour and they are not stained by counter stain saffranin.

In Gram- negative bacteria, peptidoglycan is less. Ethanol treatment causes increase in pore size of cell wall and CVI complex is removed and bacteria are decolourised and they are stained by counter stain saffranin.

3. Magnesium ribonucleate content theory: -

Magnesium ribonucleate is absent in Gram-negative bacteria and is present in Gram-positive bacteria. Hence CVI complex is removed in Gram- negative bacteria while it is not removed from Gram-positive bacteria.

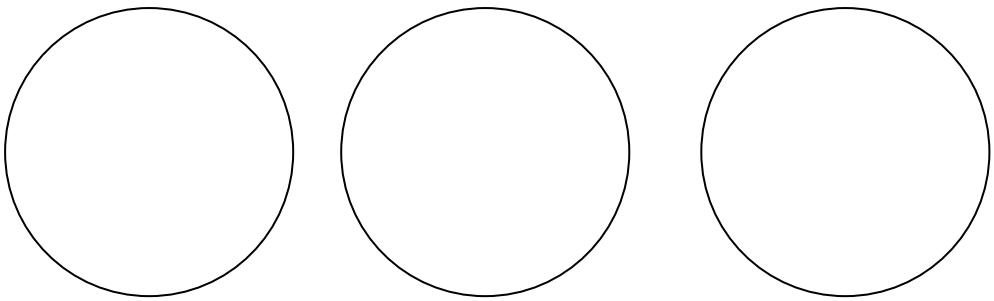
Requirements: -

1. Bacterial suspension
2. Crystal violet stain
3. Gram's iodine
4. Absolute ethanol
5. Saffranin

Procedure: -

1. On a clean grease free slide, f smear from bacterial suspension is prepared, air dried and heat fixed.
2. Crystal violet stain is applied on smear for one minute.
3. The stain is poured off and Gram's iodine is applied for 30 seconds.
4. Iodine is poured off and ethanol is applied 2 to 3 times for one minute or until violet colour stops coming out from smear.
5. Slide is washed with water and saffranin is applied for one minute.Slide is washed with water, air dried and observed under oil immersion lens.

Observations: -



1. _____ coloured and _____ shaped bacteria areobserved.

2. _____ coloured and _____ shaped bacteria areobserved.

3. _____ coloured and _____ shaped bacteria areobserved.

Result: -

1. Violet coloured _____shaped bacteria are called as Gram- positive.

2. Red coloured _____shaped bacteria are called as Gram- negative.

3. Violet coloured _____shaped bacteria are called as Gram- positive.

Conclusion: -

1. By this method we can differentiate bacteria into Gram-positive and Gram-negative.
In gram-positive bacteria lipid is less, peptidoglycan is more and Magnesium ribonucleate is present hence they appear violet in colour.

2. In gram-negative bacteria lipid is more, peptidoglycan is less and Magnesium ribonucleate is absent hence they appear red in colour

Expt. No. 06 Date:

Hanging drop technique

Aim: -

To demonstrate the motility of bacterial cell by hanging drop technique.

Theory: -

Heat fixing and staining are rather severe treatment for bacterial cell and they cause considerable changes in morphology. To avoid these changes, bacteria can be observed in their living state by means of hanging drop technique. In this method we can observe motility of bacteria. Motile bacteria have flagella.

Requirements: -

1. Suspension of motile bacteria
2. Cavity slide
3. Cover slip
4. Wax
5. Microscope

Procedure: -

1. Place a very small drop of bacterial suspension in the center of the cover slip.
2. Apply wax at the each corner of the corners of cover slip.
3. Invert the cavity of the slide over the cover slip. The cover slip adheres to the glass slide and when the cover slip is inverted, the hanging drop is seen in the cavity.
4. Bring the edges of the hanging drop into the focus with 10 X objective.
5. Turn the 40 X objective to observe the motility.

Observation: -

Motility of bacteria is observed / not observed

Result: -

Bacteria in the given suspension are motile / non motile.

Conclusion: -

By hanging drop technique we can observe the motility of bacteria.

Expt. No. 07 Date: -

Micrometry

PART I:

Micrometry is a science of measurement of size of any microscopically visible microorganism. Micrometry so actually based on the principle of physics. If we know the size of a known scale the size of unknown can be measured. The main component used in micrometry is the ocular micrometer and stage micrometer.

(a) Ocular micrometer:

The ocular micrometer is a circular disc that placed in the eyepiece. It is graduated into several small parts i.e. division marked 0_100. it is placed inside the eyepiece of the microscope. The distance varies according to the objective of the microscope. The distance is easily determined by using a stage micrometer.

(b) Stage micrometer:

The stage micrometer is a glass slides in 1 mm i.e. the scale measures only 1 mm (1000mm). Again 1 mm is divided into 10 small divisions. These divisions are equally placed.

Calibration and standard of microscope by using ocular micrometer and stage micrometer.

A microscope has the objective 10X (low power) 45X (high power) 100X (oil immersion), and the ocular lenses of 5,10and 15X. Usually, an ocular lens of 10X is in common use.

Requirements:

1. Microscope
2. Ocular micrometer
3. Stage micrometer

Procedure:

Calibration of low power lens (10X):

1. Take out the eyepiece fitted in the microscope and open the screw.
2. Put the ocular micrometer inside the ocular, tight the screw and again put into the tube of microscope.
3. Place the stage micrometer on the stage of microscope.

4. Adjust the mirror, condenser, ocular and the objective (10X, 45x, 100x) which is to be calibrated.
5. Observe under the microscope and focus properly with fine adjustment. Two different scales each of ocular and stage micrometer are visible.
6. Turn the ocular so that the parallel lines of both micrometers may become parallel.
7. Now focus the superimposed scales and coincide a line at left side and see the coincided line at the right side.
8. Count the number of ocular division coinciding between the two lines of both ocular micrometer and stage micrometer.
9. Again take at least 5_6 reading.

Result:

Calibration of high power lens (45X):

Use high [power lens (45X) of the objective and follow the steps as given above for calibration of lower lens.

Calibration of oil immersion (100X):

Put a drop of immersion oil on the stage micrometer, turn the mouth of 100X lens over it and focus with the help of the adjustment. Calculate the value of one division of ocular micrometer as described above for low power lens.

PART II:

Measurement of dimension of the given microorganism:

Once the ocular micrometer is calibrated as above, the size of any type of microorganism may be easily determined by counting the space occupied by the cells/ filament of microorganism and multiplying the value by the calibration factor of ocular micrometer.

Requirements:

1. Prepared slide of microorganism
2. Ocular micrometer
3. Stage micrometer
4. Microscope
5. Immersion oil
6. Xylene

Procedure:

- Carefully, put the calibrated ocular micrometer inside the eyepiece and place it back inside the tube of microscope.
- Put a drop of oil immersion in the center of prepared slide of bacilli where to be measured and focus properly.

- Count the number of ocular divisions that occupies the single cell of bacilli.
- Find out the accurate size of bacteria by multiplying the calibration value of ocular micrometer.

Result:

The size of the bacterium is _____

Conclusion: -

By micrometry technique we can measure the size of the microorganisms.

Expt. No. 8 i) **Date: -**

Qualitative test for Carbohydrates (Benedict's test)

Aim: - Detection of carbohydrate in the given sample by Benedict's test.

Principle:

If a suspension of copper hydroxide in alkaline solution is heated, then black cupric oxide is formed.

$Cu(OH)_2 \rightarrow CuO + H_2O$

However, if a reducing substance is present, then the rust- brown cuprous oxide is precipitated:

$2Cu(OH)_2$ ---------- $Cu_2O + H_2O + \frac{1}{2} O_2$

An alkaline solution of a copper salt and an organic compound containing alcoholic -OH is used rather than the above suspension. Under these, condition, the copper forms a soluble complex and the reagent is stable. Carbohydrates with a potentially free aldehyde or ketone group have reducing properties in alkaline solution.

Requirements:

1. Benedict's reagent
2. Glucose solution or test solution

Procedure:

1. Add five drops of the test solution to 2 ml of Benedict's reagent and place a boiling water bath for 5 min.
2. Examine the sensitivity of Benedict's test using increasing dilution of glucose.
3. Observe for rust brown colour complex.

Result: -

Brown colour formed / not formed.

Conclusion: -

The given sample contains / does not contain carbohydrate.

Expt. No. 8 ii) **Date: -**

Qualitative test for Protein (Biuret test)

Aim: Detection of protein in the sample by Biuret's method.

Principle

This is the most commonly used method and it is Based on the fact that peptide group " CONH" of protein forms a purple color with copper ions in an alkaline medium. Peptide bond is formed between two amino acids as a result of dehydration reaction i.e. removal of water molecule between carboxylic group of one amino acid and the amino group of adjacent amino acid. Since all proteins e.g. albumin, casein, gelatins, peptones enzymes contains peptide bonds.

Biuret method is fairly specific and there are little interferences during the reaction if substances like reducing sugars, if any, present. Biuret method is not only used to detect a protein but also used for quantitative estimations calorimetrically. Proteins present in various food, dairy samples, bacteria, fungi, algae can be detected and estimated by Biuret method. The other methods for the detection of protein in a sample includes Ninhydrin test, Million's test.

Requirements

1. A sample containing protein
2. Biuret reagent.

Composition – Dissolve 3g of $CuSO_4$ and 9g of sodium potassium tartarate in 500ml of 0.2 N NaOH solutions.

To this solution add 5 g of KI and the volume is made up to 1 liter with 0.2 N NaOH.

Procedure:

1. Take approximately 1 ml of the given sample.
2. Add 2 ml of biuret reagent; shake the content of the tube.
3. If pink color develops, it indicates presence of protein in a given sample.
4. The color of the biuret reagent is blue.

Result: After adding biuret reagent in the given sample, purple color developed / not developed.

Conclusion:

Purple color developed due to interaction between the copper ions present in the reagent and the peptide bonds of the protein in the given sample. Such a complex caused the change of color from blue to purple, which confirmed the presence of protein in the given sample.

Expt. No. 8 iii) **Date: -**

Qualitative test for DNA (Diphenylamine test)

Aim: - Detection of DNA by Diphenylamine test.

Principle:

When DNA is treated with diphenylamine under acid condition, a blue color compound is formed with a sharp absorption maximum at 595 nm. This reaction is given by 2- deoxypentoses in general and is not specific for DNA. In acid solution, the straight chain fore of deoxypentose is converted to the highly reactive beta hydroxyevulinaldehyde that reacts with diphenylamine to give blue complex.

Requirements:

1. DNA sample
2. Buffered saline
3. Diphenylamine reagent
4. Boiling water bath.

Procedure:

1. Dissolve 10 mg of the nucleic acid in 50 ml of the buffered saline, remove 2ml and add 4 ml of diphenylamine reagent.
2. Heat in boiling water bath, for 10 min, cool and read spectrometrically.

Result: -

Blue colour is developed / not developed. The given sample contains / does not contain DNA.

Expt. No. iv) **Date: -**

Qualitative test for RNA (Orcinol test)

Aim: Detection of RNA by Orcinol test.

Principle:

This general reaction for pentoses and depends on the formation of furfural when the pentoses is heated with concentrated hydrochloric acid. Orcinol reacts with the furfural in the presence of ferric chloride as a catalyst to give green color. Only the purine nucleotide gives any significant reaction.

Requirements:

1. RNA sample
2. Orcinol reagent
3. (Dissolve 1 g of ferric chloride in 1 liter of concentrated HCL and add 35 ml Of 6 % w/v orcinol in alcohol.).
4. Boiling water bath.

Procedure:

1. Mix 2 ml of the nucleic acid solution with 3 ml of orcinol reagent.
2. Heat on a boiling water bath for 20 min. cool and determine the extinction at 665 nm an orcinol blank.

Result: -

Green colour developed / not developed. The given sample contains / does not contain RNA.