Practical Manual
BT513P: Principles of Biochemical Engineering

Department of Biological Sciences
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Contents

Experiment No. 1:

Sterilization through Physical method.

Sterilization through Physical method.

Materials and Equipment Required

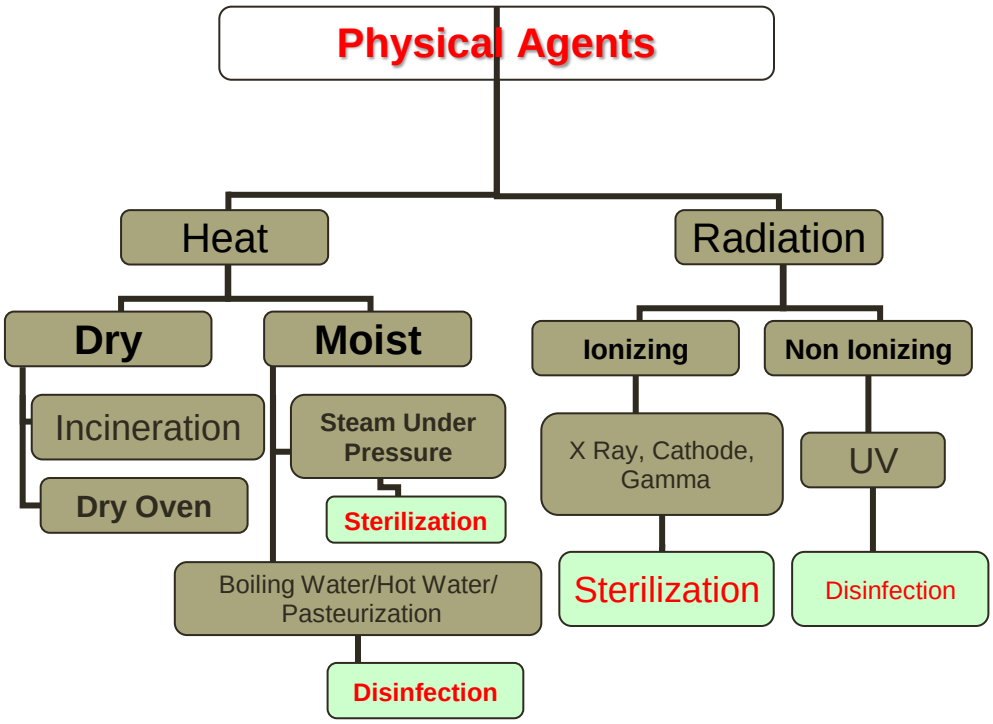
Forceps, Inoculating loop, Glass petri plates, Glass pipettes, Spirit Lamp, 70% ethanol
Hot Air oven, Laminar Flow Hood, Autoclave

Sterilization

Sterilization means the complete destruction of all the micro-organisms including spores, from an object or environment. It is usually achieved by heat or filtration but chemicals or radiation can be used.

Disinfection

Disinfection is the destruction, inhibition or removal of microbes that may cause disease or other problems e.g. spoilage. It is usually achieved by the use of chemicals.



Physical methods of sterilization

i. Sterilization by dry heat

- Dry heat kills by oxidation effects.
- The apparatus employed for dry heat sterilization is 'Hot Air Oven'.
- A temperature of about 160^{o}C for 2h is sufficient for sterilization.
- Used to sterilize glassware such as petri dishes and pipettes as well as oils, powders.

Hot air oven

ii. Incineration/flaming

- 'Destruction of microorganisms by burning'.
- Used to sterilize inoculating loop, spatulas and forceps.
- Incineration is an effective way to sterilize and dispose off contaminated paper cups, bags and dressings.

iii. Moist heat sterilization

- Kills microorganisms primarily by the coagulation of proteins (denaturation), which is caused by breakage of the hydrogen bonds that hold the proteins in their three dimensional structure.

- **Boiling**
  - An insufficient method to achieve sterilization
  - Kills vegetative cells within 30 min.
  - Endospores and some viruses, however, are not destroyed
  - Mostly used to sanitize baby bottles.

  Limitations

  - Does not kill many bacterial spores, e.g., *C. tetani* and anthrax bacilli

- Does not penetrate well into dirt, blood clots, and other protective materials which may harbor microbes.

Steam under pressure

- Heat in the form of saturated steam under pressure is the most practical and dependable agent for sterilization
- Steam under pressure provides temperatures above those obtainable by boiling.
- It has the advantages of rapid heating, penetration and moisture in abundance, which facilitates the coagulation of proteins.
- The laboratory apparatus designed to use steam under regulated pressure is called an **autoclave.**
- It is essentially a double jacketed steam chamber equipped with devices which permit the chamber to be filled with saturated steam and maintained at a desired temperature and pressure for any period of time.
    - ➢ It is absolutely essential that the air in the chamber be completely replaced by saturated steam. If air is present, it will reduce the temperature obtained within the chamber substantially below which would be realized if pure saturated steam were under the same pressure. It is not pressure that kills the organisms but the temperature of the steam.
    - Generally, but not always, the autoclave is operated at a pressure of approximately 15 psi, 121^{o}C for 15 min.
- Used to sterilize media, solutions, discarded cultures and contaminated materials.

Table: Temperature of steam under pressure

Sr. No.	Steam pressure (psi)	Temperature (^{o}C)
1.	0	100
2.	5	109
3.	10	115
4.	15	121.5
5.	20	126.5

Fig: Simple laboratory Autoclave

Fractional sterilization (Tyndallization)

- Used for the sterilization of microbiological media, solutions of chemicals, and biological materials that cannot be heated above 100^{o}C without being damaged.
- This method involves heating the material at 100^{o}C and holding it there for 15 minutes for three successive days with incubation periods in between. Resistant spores germinate during the incubation periods; on subsequent exposure to heat, the vegetative cells will be destroyed.
- An apparatus known as the **Steam Arnold** is used for this technique; however, it is also possible to operate an autoclave with free-flowing steam for this purpose.

Advantages

- Effective method
- Can be used for sterilizing items that are destroyed by autoclaving like plant seeds

Limitations

- Not considered as completely reliable method
- Only works on the media that supports the growth of bacteria from the sprouting spores.

Pasteurization

Milk, cream and certain alcoholic beverages (beer and wine) are subjected to a controlled heat treatment called pasteurization which kills microorganisms of certain types but does not destroy all organisms. Pasteurized milk is not sterile milk.

1. **Thermization**

- Milk is heated between 57°C to 68°C and holded for 15 minutes
- Targets pathogenic bacteria while leaving the good bacteria in the product.
- Structure and taste of the milk is not changed

2. **Batch pasteurization/ low-temperature long time (LTLT) pasteurization**

- Milk is heated to 63°C for 30 minutes
- Milk protein structure and taste is changed due to the extended holding time

3. **Flash pasteurization/ high-temperature short time (HTST) pasteurization**

- Milk is heated between 72°C to 74°C for 15 to 20 seconds.

4. **Ultra-high temperature (UHT) pasteurization**

- Milk is heated between 135°C to 140°C for 2 to 4 seconds.
- The heat kills all the vegetative forms of bacteria and the shelf life of milk is increased up to 9 months.

ii. <u>Sterilization by radiation</u>

a) **Ionizing radiations**

- Short wavelength, high-intensity radiation used to destroy microorganisms
- Cause damage to the genetic material of the cell (DNA or the RNA)
- Include gamma rays, X-rays or high energy electron beams
- Have short wavelength
- Have high penetrating power.
- X-rays have a wavelength ranging from 10 picometers to 10 nanometers
- Natural sources of gamma rays on Earth include gamma decay from naturally occurring radioisotopes such as potassium-40, and also as a secondary radiation from various atmospheric interactions with cosmic ray particles.
- X-rays are a form of electromagnetic radiation, similar to visible light. x-rays have higher energy and can pass through most objects, including the body.
- X-rays are produced when charged particles of sufficient energy hit a material. The production of X-rays relates to energy changes in an electron, such as moving from a higher energy level to a lower one, causing the excess energy to be released.

- Ionizing radiation are used for the sterilization of pharmaceuticals and disposable dental and medical supplies, such as plastic syringes, surgical gloves, suturing materials and catheters.

b) **Nonionizing radiation**

- Non-ionizing radiation refers to any type of electromagnetic radiation that does not carry enough energy per quantum (photon energy) to ionize atoms or molecules—that is, to completely remove an electron from an atom or molecule.
- Nonionizing radiation has a wavelength longer than ionizing radiation. The best example of non-ionizing radiation is ultraviolet (UV) light.
- Ultraviolet (UV) light consists of light of wavelengths between 10 to 400 nm, but wavelength in the 200 nm range are most effective in killing microorganisms. But according to some books wavelength between 260 – 265 nm is most effective.
- Ultraviolet light effectively reduces the microbial population where direct exposure takes place.
- It is used to limit airborne or surface contamination in a hospital room, morgue, pharmacy, toilet facility, or food service operation.
- UV lamps are used to disinfect and sterilize surfaces in industries and hospitals, restaurants, schools, commercial and industrial properties and public places. Also used in pools and spas, wastewater treatment, drinking water and aquaculture etc.
- Sterilize surgical equipment, operating rooms, patient rooms, patient beds, surfaces, restrooms

Experiment No. 2

Sterilization through Chemical method

Materials and Equipment Required

70% Ethyl alcohol, Inoculating loop, Bleach

Different chemicals (liquids/gases) are used for sterilization.

Ethylene oxide gas

- Cause denaturation of proteins.
- Kills all microbes and endospores and endospores but requires a lengthy exposure period of 4 to 18 h.
- It is toxic and explosive in its pure form, so it is usually mixed with a nonflammable gas, such as carbon dioxide or nitrogen.
- It has high penetration power.
- Used to sterilize medical supplies and equipment.
- Used to sterilize/disinfect different types of equipment and surfaces like plastic and rubber articles, oils and some foods.

Propylene oxide and β-propiolactone

- Used in fumigation
- For sterilization, 0.2% BPL is used
- Has a rapid biocidal activity
- Very effective against viruses

❖ A disadvantage of all these gases is that they are suspected carcinogens especially β-propiolactone.

Plasma gas sterilization

- This makes use of vapors of hydrogen peroxide subjected to radio frequencies to produce reactive free radicals.
- No by-products toxic to humans are produced, and is an effective sterilant.
- Used for surgical instruments mainly those with narrow lumen such as arthroscopes and laproscopes.

Formaldehyde

- inactivates microorganisms by alkylating the amino and sulfhydral groups of proteins and ring nitrogen atoms of purine bases.
- used as a disinfectant and sterilant in both its liquid and gaseous states.

- It is sold and used principally as a water-based solution called formalin, which is 37% formaldehyde by weight.

Limitations

- produce irritating fumes and possess pungent odor even at very low levels (<1 ppm)
- suspected human carcinogen linked to nasal cancer and lung cancer

Glutaraldehyde

- Works by denaturing proteins
- Used to disinfect hospital instruments, including respiratory-therapy equipment.
- When used in 2% solution (Cidex), it is bactericidal, tuberculocidal and virucidal in 10 min, and sporicidal in 3-10h.
- Glutaraldehyde is one of the few liquid chemical disinfectants that can be considered a sterilizing agent.

Peroxygens

- Exert antimicrobial activity by oxidizing cellular components of the treated microbes.

a) Hydrogen peroxide

- Found in many household medicine cabinets and in hospital supply rooms.
- It does effectively disinfect inanimate objects, an application in which it is even sporicidal, especially at elevated temperatures.
- Food industry is increasing its use of hydrogen peroxide for aseptic packaging.

b) Peracetic acid

- One of the most effective liquid chemical sporicides available and is considered a sterilant.
- It is generally effective on endospores and viruses within 30 min and kills vegetative bacteria and fungi in less than 5 min.
- Used for the disinfection of food-processing and medical equipment because it leaves no toxic residues and is minimally affected by the presence of organic matter.

Alcohols

- Alcohols work by denaturing proteins.
- The types of alcohol used in disinfection are ethanol (80%), propanol (60%), and isopropanol (70%)

- Effective against bacteria and fungi, less so against viruses. They do not kill bacterial spores.

Uses

- Used for surgical and hygienic disinfection of the skin and hands.
- recommended for sterilizing medical and surgical materials
- to disinfect oral thermometers
- to disinfect rubber stoppers of medication vials or vaccine bottles

Disadvantage

- effect is not long-lasting

Chlorine and Chlorine Compounds

- inactivates microorganism by oxidation of sulfhydryl enzymes and amino acids; ring chlorination of amino acids; loss of intracellular contents; decreased uptake of nutrients; inhibition of protein synthesis; decreased oxygen uptake; oxidation of respiratory components; decreased adenosine triphosphate production; breaks in DNA; and depressed DNA synthesis

Uses

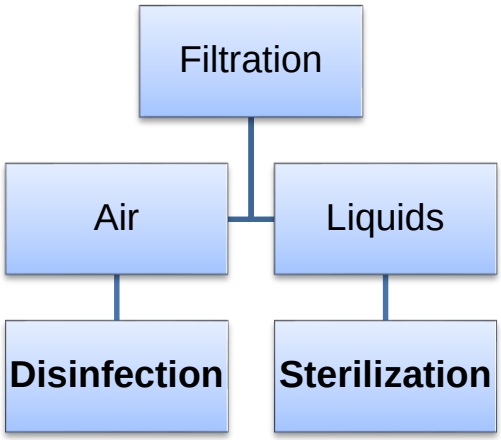
- Water purification
- Sanitation of utensils in dairy and restaurant industries
- Chloramine (0.1% to 2% solutions) for wound irrigation and dressings

Experiment No. 3

Sterilization by Filtration

Materials and Equipment Required

Membrane Filters, Laminar flow hood cabinet



Depth filters

Depth filters are a range of filters that use a porous filtration medium to hold particles in the medium, rather than just on the surface of the medium. These filters are normally used when the fluid to be filtered holds a high load of particles because they can hold a large mass of particles before becoming blocked.

a. Sintered (or fritted) glass filters

Borosilicate glass is finely powdered in a ball-mill and the particles of required size are separated. This is packed into disc mounted and heated till the particles get fused. The disc thus made have pore size of 2 mm and are used for filtration. As these are made of glass and hence do not absorb liquids during filtration. The disadvantage is that they are very brittle and break easily

They are cleaned with the help of sulfuric acid.

Applications in laboratory glassware include use in fritted glass filter items, scrubbers, or spargers.

b. Seitz filter:

These are made of asbestos or other material. They are pad like and thicker than membrane filters. They do not rupture during filtration. But the solution might get absorbed by the filter pad itself. A filter disc (originally of asbestos) with pores so fine that they will not permit passage of bacteria; solutions emerge sterile.

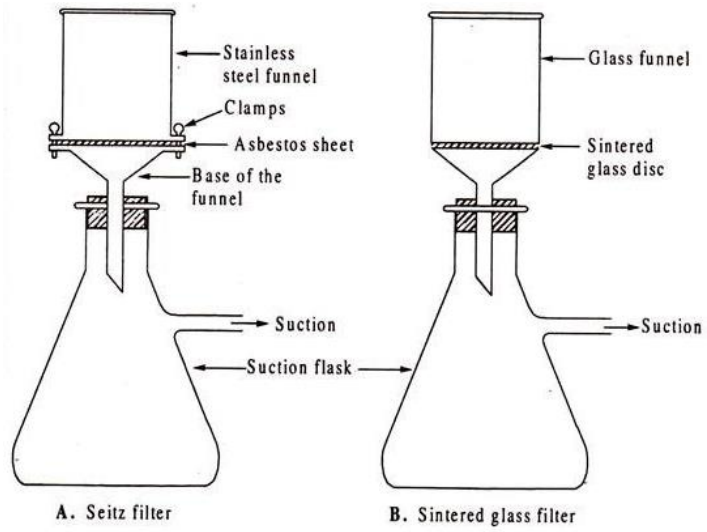


Fig: Types of Depth Filters

Membrane filters

- Membrane filters are made of cellulose-derivative (acetate or nitrate). They are very fine. They are fixed in some suitable holders.
- Nominal pore size is 0. 22 ± 0. 02 mm or less is required.
- The membranes are brittle when dry. In this condition they can be stored for years together. They become very tough when dipped in water.
- They are sterilized by autoclaving or by ethylene oxide gas. They cannot be sterilized by dry heat as they decompose above 120^0C.
- They are suitable for sterilizing aqueous and oily solutions but not for organic solvents such as alcohol, chloroform etc.
- Membrane filters are generally blocked by dirt particles and organisms. Pre-filtration (through glass-fibre paper prefilter) reduces the risks of blockage of the final filter.

➢ *Examples of membrane filters:*

MF-Millipore – it is a mixture of cellulose esters

Advantages of sterilization by filtration:

1. Thermolabile solutions can be sterilized.
2. It removes all the living microorganisms.

Disadvantages of sterilization by filtration:

1. Filters may break down suddenly or gradually on use.
2. Sterility testing is obligatory on the filtered solution.
3. Filter media may be absorbed on the filter surface.
4. Viruses are not removed by filtration.
5. Suspensions and oils cannot be sterilized by this method due to their heavy load of particulate matters and viscosity.

a) Air filters

- Air is filtered by HEPA (High efficiency particulate air) filters.
- Remove almost all microorganisms larger than about 0.3 μm in diameter.
- Air filters are used in operation theaters, rooms occupied by burn patients and safety cabinets.

Experiment No. 4

Media formulation for bacteria

Materials Required

LB, Nutrient Broth, Mannitol Salts Agar, MacConkey agar, *Salmonella Shigella* Agar, agar, Erlenmeyer flask, glass stirrer, cotton, beaker, cylinder.

Introduction

All micro-organisms require water, sources of energy, carbon, nitrogen, mineral element and vitamin plus oxygen in their growth medium. On a small scale, it is simple to device a medium containing pure compounds, but the resulting medium although satisfy the growth, may be unsuitable for use in a large scale process.

The essential part of media designing is to be considering the stoichiometry of growth and formation of desired product which is given below:

Carbon and energy source + Nitrogen source + O2 + Other requirements ----> Biomass + Products + CO2 + H2O+ Heat

Essential characteristics of formulated media

1. It should produce the maximum yield, maximal concentration with maximal rate of production of desired biomass, of consistent quality which is always readily available.
2. It should also have minimal problems during media designing.
3. It should have minimal problems during recovery of desired product/ biomass especially during aeration and agitation, extraction, purification and waste treatment.

Basic media constituents

1. Water

It is major component of all fermentation media. Following factors need to be considered about water for media preparation

- pH
- Dissolve salt

Mineral water content of water is very important in brewing, and most critical in the mashing process, and historically influenced the siting of breweries and the type of beer produced.

2. Energy Sources

Energy for growth comes from either the oxidation of medium components or from light. Most industrial micro-organisms are chemo-organotrophs, therefore the commonest sources of energy are the carbon sources.

3. Carbon sources

Carbon requirement for the medium is normally provided by:

- **Sucrose**: Sugarcane, sugar beet molasses
- **Glucose**: Corn sugar, starch, cellulose
- **Lactose**: Milk whey
- **Fats**: Vegetable oil
- **Starch**: Maize grains, cereals, potatoes and cassava
- **Hydrocarbons**: Petroleum fractions

4. Nitrogen Sources

Microorganisms generally can use inorganic or organic N.

- Inorganic sources: ammonia, ammonium salts
- Organic sources: amino acid, proteins and urea, Corn steep liquor, Yeast extract, Peptones, Soya bean meal.

5. Oxygen sources

Oxygen is always provided in water.
Some organisms require molecular oxygen as terminal oxidizing agents to fulfill their energetic needs through aerobic respiration. These organisms are obligatorily aerobic.
For obligate anaerobes molecular 02, is a toxic substance.
Some organisms are facultative anaerobes and can grow with or without molecular 02.

Types of media and culturing

Culture media contains nutrients and physical growth parameters necessary for microbial growth. All microorganisms cannot grow in a single culture medium and in fact many can't grow in any known culture medium.

Bacterial culture media can be distinguished on the basis of **composition, consistency and purpose.**

Classification of culture media used in Microbiology laboratory on the basis of consistency

1. **Solid medium**
   Solid medium contains agar at a concentration of 1.5-2.0% or some other, mostly inert solidifying agent. Solid medium has physical structure and allows bacteria to grow in physically informative or useful ways (e.g. as colonies or in streaks). Solid medium is useful for **isolating bacteria** or for determining the colony characteristics of the isolate.
2. **Semisolid media**

They are prepared with agar at concentrations of 0.5% or less. They have soft custard like consistency and are useful for the cultivation of **microaerophilic bacteria** or for **determination of bacterial motility.**

3. **Liquid (Broth) medium**
   These media contains specific amounts of nutrients but don't have trace of gelling agents such as gelatin or agar. Broth medium serves various purposes such as propagation of large number of organisms, fermentation studies, and various other tests. e.g. sugar fermentation tests, **MR-VR broth.**

Classification of culture media based on the basis of composition

1. Synthetic or chemically defined medium
   A chemically defined medium is one prepared from purified ingredients and therefore whose exact composition is known.
2. Non synthetic or chemically undefined medium

Non-synthetic medium contains at least one component that is neither purified nor completely characterized nor even completely consistent from batch to batch. Often these are partially digested proteins from various organism sources. Nutrient broth, for example, is derived from cultures of yeasts.

Synthetic medium may be simple or complex depending up on the supplement incorporated in it. A simple non-synthetic medium is capable of meeting the nutrient requirements of organisms requiring relatively few growth factors whereas complex non-synthetic medium support the growth of more fastidious microorganisms.

Classification of Bacterial Culture Media based on the basis of purpose/ functional use/ application

Many special purpose media are needed to facilitate recognition, enumeration, and isolation of certain types of bacteria. To meet these needs, numerous media are available.

1. General purpose media/ Basal media

Basal media are basically simple media that supports most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar are considered as basal medium. These media are generally used for the primary isolation of microorganisms.

e.g. Ingredients for nutrient agar

- 10g NaCl
- 10g Tryptone
- 5g Yeast Extract
- 15 g Agar
- and dH_2O to 1 liter

Nutrient Agar

2. Enriched medium (Added growth factors):
Addition of extra nutrients in the form of blood, serum, egg yolk etc, to basal medium makes them enriched media. Enriched media are used to grow nutritionally exacting (fastidious) bacteria. Blood agar, chocolate agar, Loeffler's serum slope etc are few of the enriched media. Blood agar is prepared by adding 5-10% (by volume) blood to a blood agar base. Chocolate agar is also known as heated blood agar or lysed blood agar**.**

Composition of blood agar

- 0.5% Peptone
- 0.3% beef extract/yeast extract
- 1.5% agar
- 0.5% NaCl
- Distilled water

(Since Blood Agar is made from Nutrient Agar, above is the composition of Nutrient Agar)

- 5% Sheep Blood
- pH should be from 7.2 to 7.6 (7.4)

Blood Agar

3. Selective and enrichment media are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria. While selective media are agar based, enrichment media are liquid in consistency. Both these media serve the same purpose. Any agar media can be made selective by addition of certain inhibitory agents that don't affect the pathogen of interest. Various approaches to make a medium selective include **addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these.**

a. Selective medium

Principle: Differential growth suppression

Selective medium is designed to suppress the growth of some microorganisms while allowing the growth of others. Selective medium are agar based (solid) medium so that individual colonies may be isolated.

Examples of selective media include:

Mannitol Salt Agar (MSA), used to recover *S.aureus* contains 7.5 - 10% NaCl. Ingredients for MSA are given below;

- 5.0 g/L enzymatic digest of casein.
- 5.0 g/L enzymatic digest of animal tissue.
- 1.0 g/L beef extract.
- 10.0 g/L D-mannitol.
- 75.0 g/L sodium chloride.
- 0.025 g/L phenol red.
- 15.0 g/L agar.
- pH 7.4 ± 0.2 at 25°C.

Fig. Mannitol Salt Agar grows halophilic (salt-loving) bacteria. Pathogenic *Staph* is growing on left (yellow side) and normal flora *Staph* growing on right (pink).

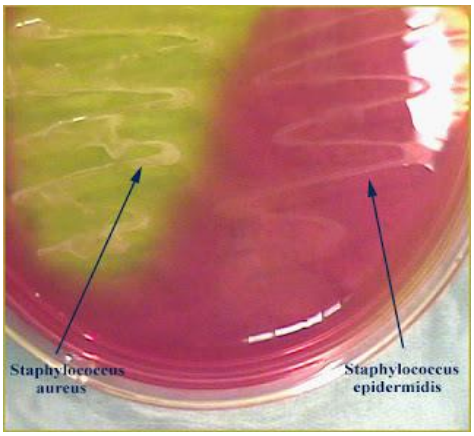
MacConkey's Agar used for **Enterobacteriaceae** members contains bile salt that inhibits most gram positive bacteria.
Composition of MacConkey agar is given below;

1. Peptone – 17 g.
2. Proteose peptone – 3 g.
3. Lactose – 10 g.
4. Bile salts – 1.5 g.
5. Sodium chloride – 5 g.
6. Neutral red – 0.03 g.
7. Crystal violet – 0.001 g.
8. Agar – 13.5 g.
9. d.H_2O – 1liter

4. Differential medium

Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony color. Various approaches include incorporation of dyes, metabolic substrates etc, so that those bacteria that utilize them appear as differently coloured colonies. Such media are called differential media or indicator media. Differential media allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies.

Examples of differential media include:
Mannitol salts agar (mannitol fermentation = yellow)
MacConkey agar (lactose fermenters, pink colonies (*E. coli*) whereas non- lactose fermenter produces pale or colorless colonies.



MacConkey agar:

It differentiates the gram-negative bacteria based on their lactose metabolism. The lactose fermenting bacteria, such as *Escherichia coli, Klebsiella spp, Citrobacter,* and *Enterobacter* forms pink-red colonies, while lactose non-fermenters, like *Salmonella, Shigella, Proteus, Providencia, Pseudomonas,* and *Morganella* form pale or colorless colonies.

Blood agar

In blood agar, three types of blood cell lysis or hemolysis are observed: alpha, beta, and gamma hemolysis. It allows the growth of many microorganisms, however, their ability to lyse blood cells differs, and this helps to distinguish the bacterial colonies.
S. pyogenes completely lyse blood cells (beta hemolysis), thus causing total clearing of the media/ yellowish zone around its colonies.
S. pneumoniae partially lyse red blood cells, resulting in a greenish-colored medium, while gamma hemolytic microorganisms like *Enterococcus faecalis, Staphylococcus saprophyticus,* and *Staphylococcus epidermidis*, can't lyse red blood cells, thus causing no color change in the medium.

Procedure

1. Weigh appropriate amount of NA/LB/MSA/MacConkey agar/ SS agar separately as per manufacturer's instructions to prepare 100 ml of each media.
2. Dissolve the media in small amount of distilled water and raise the volume of the media to 100 ml.
3. Dissolve agar by heating on the hot plate.
4. Transfer the individual media into Erlenmeyer flasks. Cotton plug the flasks and sterilize in an autoclave at 121^{o}C, 15 psi for 15 min.
5. After sterilization, pour 10-15 ml of each agar medium in sterilized petri plates aseptically.
6. Allow the medium to solidify.

7. Add 0.1 ml from 10^{-2} dilution of given sample (soil/milk/juice) on petri plate and spread evenly.
8. Incubate the plates in an incubator at 37^{o}C for 24-48 h.
9. Observe the bacterial growth and zone on differential media and record observations.
10. For LB medium, add a single bacterial colony aseptically in the flask and incubate at 37^{o}C for 24 h in a shaking incubator. The turbidity in the flask shows bacterial growth.

Experiment No. 5

Initiation of bacterial culture in a shake flask

Materials and Equipments Required

LB/NB, Erlenmeyer flask, glass stirrer, cotton, Erlenmeyer flask, weighing balance, shaking incubator, autoclave

Procedure

1. To prepare 100 ml of LB, weigh out the following and add in to 50 ml of distilled water, adjust the pH to 7.0 with 0.1 N phosphoric acid/0.1 N NaOH and make up the volume to 100 ml with distilled water.

    - 1 g NaCl
    - 1 g Tryptone
    - 0.5 Yeast Extract

2. Transfer medium to 250 ml Erlenmeyer flask and cotton plug it.
3. Sterilize by autoclaving at 15 psi, 121°C for 15 minutes.
4. After sterilization, allow the medium to cool to room temperature.
5. Inoculate the flask with a loopful of pure bacterial culture aseptically.
6. Incubate the flask at 37^{o}C for 24 h in a shaking incubator (150 rpm). After 24 h, the turbidity in the flask shows bacterial growth.

Experiment No. 6

Initiation of bacterial culture in a Fermenter

Materials and Equipments Required

LB, Erlenmeyer flask, glass stirrer, cotton, beaker, cylinder, weighing balance, autoclave, fermenter, Shaking incubator

Procedure

1. Prepare suitable media (basal media/fermentation media) for the growth of the bacteria.
2. Add the media in the vessel of the fermenter. Assemble the fermenter accessories. Sterilize the fermenter vessel along with its accessories in an autoclave.
3. For inoculum, prepare the LB medium, pour in suitable Erlenmeyer flask and cotton plug it. Sterilize the medium in an autoclave. Inoculate the LB medium with pure culture. After inoculation, place the flask in a shaking incubator at 37°C for overnight. The overnight grown culture will be used as inoculum.
4. In fermenter, cells are grown to a particular density while maintain the environmental conditions constant depending upon the organism used (37^{o}C, pH: 7:0). When the optimal growth is obtained, the biomass is harvested and the fermenter is cleaned for the next batch.

Experiment No. 7

Bacterial Growth Curve

Materials and Equipment Required

LB broth, Erlenmeyer flask, test tube, cotton, beaker, cylinder, stirrer, Spectrophotometer, shaking incubator

Procedure:

1. Prepare LB medium, add 100 ml in an Erlenmeyer flask and 5 ml in test tube (blank).
2. Sterilize the medium in an autoclave.
3. Inoculate the flask with overnight grown bacterial culture (0.5 ml).
4. Adjust the wavelength of the spectrophotometer to 600 nm. Set 'zero' with blank.
5. Take 5 ml from the inoculated flask and take the absorbance.
6. Incubate the flask at 37°C (150 rpm). Take the sample from the incubated flask after every 20 min and measure absorbance after setting zero with blank.
7. Record the value of OD with respect to time, until decline phase is observed.
8. Plot graph taking optical density on y-axis and time on x-axis.

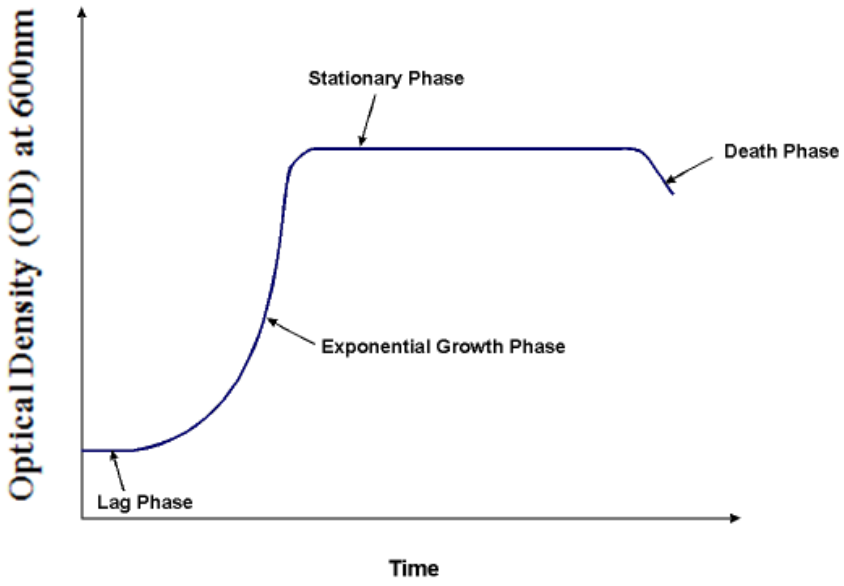


Fig: Bacterial Growth Curve (OD vs Time)

Observations

Incubation Time (min)	OD at 600 nm
20	
40	
60	
80	
100	
120	
140	

Experiment No. 8
Medium formulation (fungi)

Materials and Equipments Required

PDA, erlenmeyer flask, cylinder, beaker, hot plate, pH meter, weighing balance, autoclave, incubator, Laminar Flow Hood

Procedure (Preparation of PDA)

1. Add 3.9 grams of premixed PDA in 50 ml distilled water. Adjust the pH to 5.5 with 0.1 N phosphoric acid.
2. Heat to boiling to dissolve the medium completely. Make up the volume to 100 ml with distilled water.
3. Transfer medium to 250 ml Erlenmeyer flask and cotton plug it.
4. Sterilize by autoclaving at 15 psi, 121°C for 15 minutes.
5. After sterilization, allow the medium to cool at 60^{o}C. Add antibiotic to prevent bacterial growth. Pour 10-15 ml of PDA in sterilized petri plates aseptically.
6. Allow the media in the petri plates to solidify.
7. Add 0.2 ml from 10^{-2} dilution (soil sample) and spread with the help of spreader.
8. Incubate the plates at 30^{o}C for 3 days.
9. Observe fungal growth and identify fungi based on morphology.

Experiment 9

Medium formulation (Yeast)

Materials and Equipments Required

YPD, Yeast Extract, peptone, dextrose, erlenmeyer flask, cylinder, beaker, hot plate, pH meter, weighing balance, autoclave, incubator, Laminar Flow Hood, shaking incubator

Procedure

YPD

1. Add peptone 2.0 grams, Yeast extract 1.0 g and dextrose 2.0 g in 50 ml distilled water. Adjust the pH to 5.0 with 0.1 N phosphoric acid.
2. Make up the volume to 100 ml with distilled water.
3. Transfer medium to 250 ml Erlenmeyer flask and cotton plug it.
4. Sterilize by autoclaving at 15 psi, 121°C for 15 minutes.
5. After cooling of the medium to room temperature, add loopful of yeast culture.
6. Incubate the flask in a shaking incubator at 30^{o}C for 24-48 h.
7. Turbidity in flask shows yeast growth.

YPD agar

1. Add peptone 2.0 grams, Yeast extract 1.0 g, dextrose 2.0 g and agar 1.6 g in 50 ml distilled water. Adjust the pH to 5.0 with 0.1 N phosphoric acid.
2. Heat to boiling to dissolve the medium completely. Make up the volume to 100 ml with distilled water.
3. Transfer medium to 250 ml Erlenmeyer flask and cotton plug it.
4. Sterilize by autoclaving at 15 psi, 121°C for 15 minutes.
5. Pour 10-15 ml of PDA in sterilized petri plates aseptically.
6. Allow the media in the petri plates to solidify.
7. Add 0.2 ml from 10^{-2} dilution (soil sample) and spread with the help of spreader.
8. Incubate the plates at 30^{o}C for 24-48 h days.
9. Observe yeast growth.

Experiment 10

Production of enzyme (alpha amylase) by submerged fermentation and estimation of alpha amylase activity

Materials Required

Beaker, Stirrer, 250 ml Erlenmeyer flask, cylinder, soluble starch, KNO_3, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2$, $FeCl_3$

Procedure

1. Add 50 ml of fermentation medium (g/L: soluble starch, 10; KNO_3, 0.5; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; $FeCl_3$ pH: 7.0) in 250 ml Erlenmeyer flask.
2. Cotton plug the flask and sterilize the flask in an autoclave at 121°C, 15 psi for 15 minutes.
3. Cool the flask at room temperature.
4. Add one ml of the inoculum (bacterial cell suspension) to the flask.
5. Incubate the flask at 37°C for 48 h.
6. After incubation, centrifuge the contents of the flask at 6000 rpm for 15 minutes.
7. Use the supernatant for the estimation of enzyme (alpha amylase).

Estimation of Alpha amylase

Standard curve for Maltose

Take70 ml of distilled H_2O and dissolve 100 mg maltose and made the volume up to 100 ml. Take ten labelled test tubes and then prepare ten dilutions containing maltose (0.1-1.0 mg) with final volume (2 ml) using distilled water. Then add 2 ml of DNS reagent in each test tube. Prepare blank by adding 2 ml of DNS and 2 ml distilled water. Put the test tubes into boiling water for 5 min and cool at room temperature. Add 6 ml distilled water to the test tube to make total volume 8 ml. take OD at 550 nm using spectrophotometer after setting zero with blank. Draw graph taking maltose concentration on X axis and absorbance along Y axis.

α-amylase Assay

The activity of reducing sugar and α-amylase can be estimated by using DNS method following protocol of Stegbauer and Rick (1974). Add 1 ml of 1% starch solution (pH 7/PH 5.5) to a test tube and add 1 ml of enzyme extract (Sample). For control, add 1 ml of starch solution. Incubate the test tubes at 37°C/30°C for 10 minute in water bath. After incubation, added 1 ml enzyme extract in control tube and then added 1 ml DNS in both test tubes and boil on hot plate for 5 minutes. After boiling, add 6 ml DH_2O and observe the absorbance at 550 nm using spectrophotometer. One unit of α-amylase is defined as "The quantity of enzyme required to release one µmol of maltose per min under the study condition".

$$U/ml/min= X \times 1000/10 \times 342.30$$

where

X is the concentration of standard (mg/mL)

Reference

Rick, W., & Stegbauer, H. P. (1974). α-Amylase measurement of reducing groups. In *Methods of enzymatic analysis* (pp. 885-890). Academic Press.

Experiment No. 11

Production of enzyme (alpha amylase) by solid state fermentation

Materials Required

Beaker, Stirrer, 250 ml Erlenmeyer flask, cylinder, wheat bran, Peptone, yeast extract, Starch, NaCl, $CaCl_2$ and $MgSO_4$

Procedure

1. Add ten grams of solid substrate such as wheat bran in 250 ml Erlenmeyer flask.
2. Moisten the substrate with 10 ml of suitable diluent (Peptone (10 g); yeast extract (4 g); Starch (20 g); NaCl (0.5 g); $CaCl_2$ (0.2 g) $MgSO_4$ (0.5 g); Distilled water up to 1000 ml).
3. Cotton plug the flask and sterilize the flask in an autoclave at 121°C, 15 psi for 15 minutes.
4. Cool the flask at room temperature.
5. Add one ml of the inoculum (bacterial cell suspension/ fungal conidial suspension) to flask.
6. Incubate the flask at 30/37°C for 48-72 h.
7. After fixed period of incubation, add 100 ml of distilled water to each flask containing fermented bran.
8. Place the flask in incubator shaker at 160 rpm for one hour.
9. After one hour, filter the contents of the flasks and use the filtrate for the estimation of enzyme (alpha amylase).