Practical Manual

Principles of Biochemical Engineering (Bio204)

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Contents

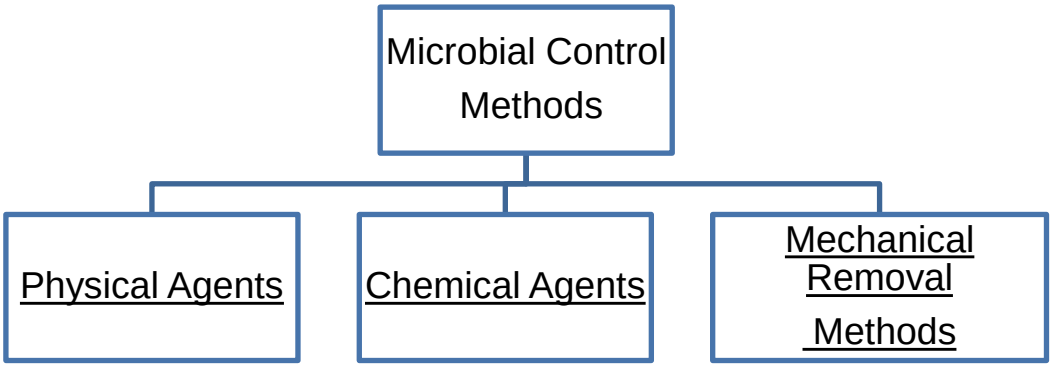
Experiment No. 1:

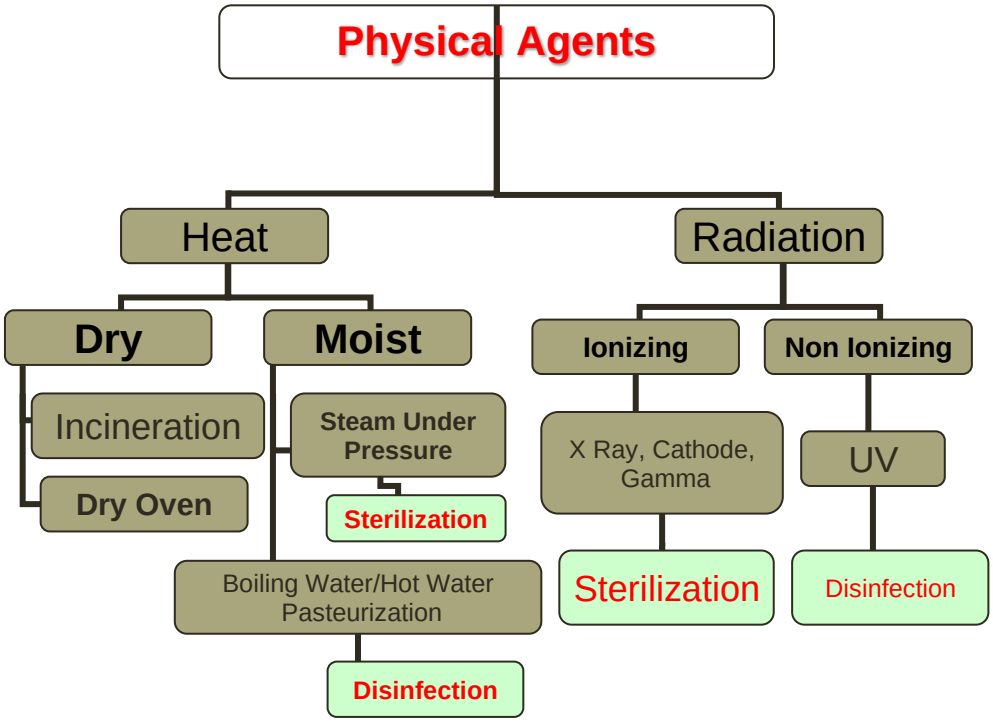
Sterilization through Physical method.

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 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 Agents
Heat
Radiation
Dry
Moist
Ionizing
Non Ionizing
Incineration
Steam Under Pressure
X Ray, Cathode, Gamma
UV
Dry Oven
Sterilization
Sterilization
Disinfection
Boiling Water/Hot Water Pasteurization
Disinfection

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.

a. **Boiling**

- Kills vegetative forms of bacterial pathogens, almost all viruses, fungi and their spores within about 10 min. Endospores and some viruses, however, are not destroyed this quickly.
  - Hepatitis virus: Can survive up to 30 minutes of boiling.
  - Endospores: Can survive up to 20 hours or more of boiling.

Boiling is therefore not always a reliable sterilization procedure.

- Mostly used to sanitize baby bottles.

b. **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)

- Some microbiological media, solutions of chemicals, and biological materials cannot be heated above 100^{o}C without being damaged. If, however, they withstand the temperature of free flowing steam (100^{o}C), it is possible to sterilize them by fractional sterilization (tyndallization).
- This method involves heating the material at 100^{o}C on 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.

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.

- **Holder method:** In the classical pasteurization treatment of milk, the milk was exposed to a temperature of about 63^{o}C for 30 min.
- **High-temperature short-time (HTST):** most milk pasteurized today uses higher temperatures, atleast 72^{o}C but for only 15 sec. in addition to killing pathogens, HTST pasteurization lowers total bacterial counts, so the milk keeps well under refrigeration.
- **Ultra-high-temperature treatments (UHT):** milk can also be sterilized by UHT (something quite different from pasteurization). The milk falls in a thin film through a chamber of superheated steam and reaches 140^{o}C in less than second. It is held for three

seconds in a holding tube and then cooled in a vacuum chamber, where the steam flashes off. With this process, in less than 5 sec the milk temperature rises from 74^{o}C to 140^{o}C and drops back to 74^{o}C.

ii. Sterilization by radiation:

a) **Ionizing radiation**

- Include gamma rays, X-rays or high energy electron beams.
- Gamma rays are emitted from certain radioactive elements such as cobalt, electron beams are produced by accelerating electrons to high energies in special machines.
- Have wavelength less than about 1 nm.
- Have high penetrating power.
- Ionizing radiation, especially high energy electron beams, is 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**

- Nonionizing radiation has a wavelength longer than ionizing radiation, usually greater than about 1 nm. The best example of non ionizing radiation is ultraviolet (UV) light.

- Ultraviolet (UV) light consists of light of wavelengths between 40 to 390 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.

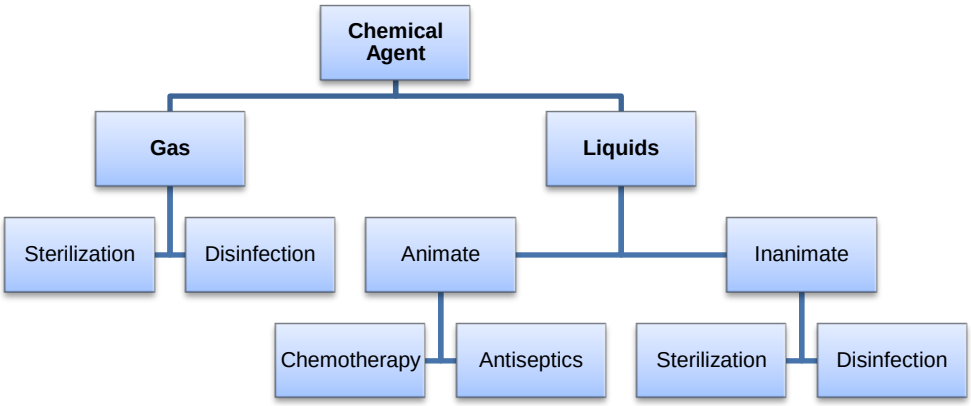
- It is used to limit airborne or surface contamination in a hospital room, morgue, pharmacy, toilet facility, or food service operation.

- A major disadvantage of UV light as a disinfectant is that the radiation is not very penetrating, so the organism to be killed must be directly exposed to the rays. Organisms protected by solids and such coverings as paper, glass, or fabric are not affected.

- Another potential problem is that UV light can damage human eyes, and prolonged exposure can cause burns and skin cancer in humans. And it may cause damage in human skin cells.

Experiment No. 2

Sterilization through Chemical method



Sterilization by gases

a) 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 plastic and rubber articles, oils and some foods.

b) Propylene oxide and β-propiolactone

- Used in fumigation
- For sterilisation 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.

c) Plasma gas sterilization

- This makes use of vapors of hydrogen peroxide subjected to radio frequencies or microwave radiation 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.

Chemical methods

i) 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.

ii) Peroxygens

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

a) Hydrogen peroxide

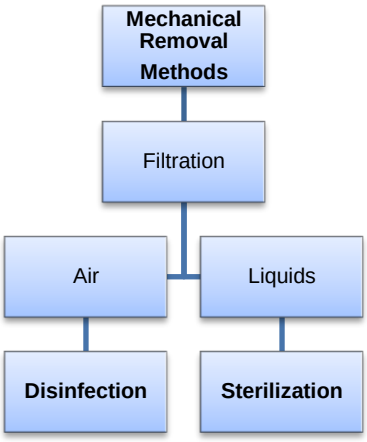
- Found in many household medicine cabinets and in hospital supply rooms.
- It does effectively disinfect inanimate objects, an application in which it is even sporicidal, especially at elevated temperatures.
- Food industry is increasing its use of hydrogen peroxide for aseptic packaging.

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.

Experiment No. 3

Sterilization through Mechanical method



iii. Sterilization by filtration:

This method is used for sterilizing thermolabile solutions, which will otherwise be degraded by other conventional heating methods. The drug solutions are passed through the sterile bacteria proof filter unit and subsequently transferring the product aseptically into the sterile containers which are then sealed.

Depth filters

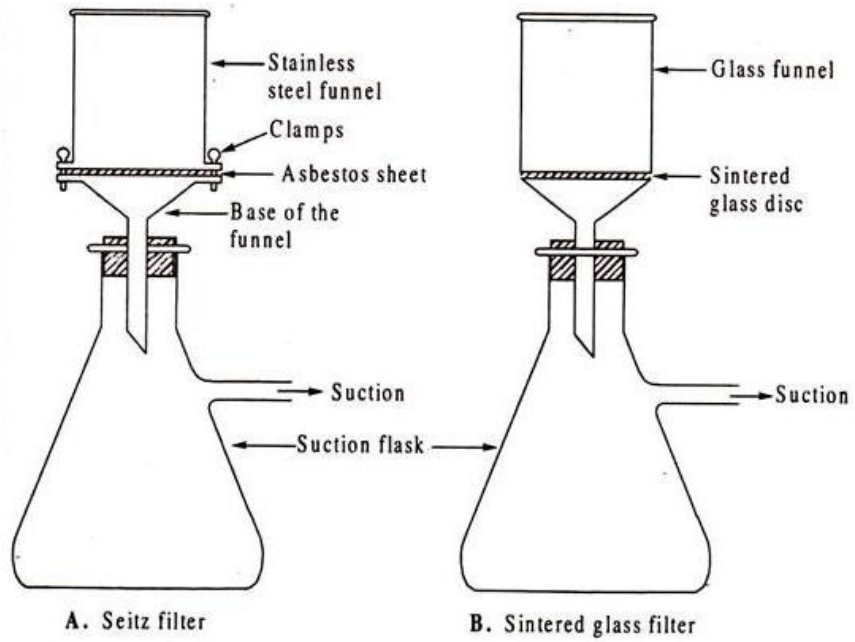
Depth filters are the variety of filters that use a porous filtration medium to retain particles throughout the medium, rather than just on the surface of the medium. These filters are commonly used when the fluid to be filtered contains a high load of particles because, relative to other types of filters, they can retain a large mass of particles before becoming clogged.

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.

b. Sietz 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.

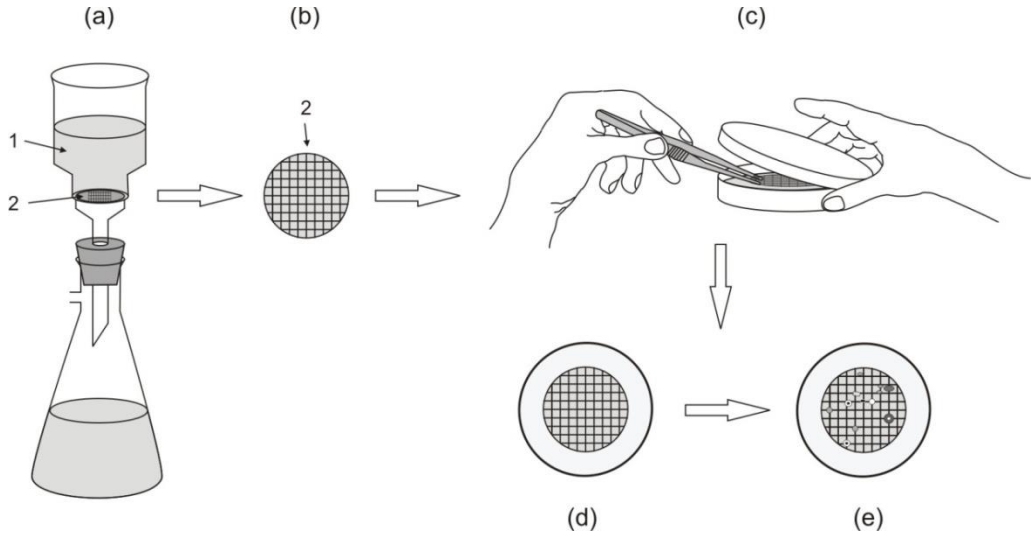


Membrane filters

- 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



a) Air filters

- Air is filtered by HEPA (High efficiency particulate air filters) 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.

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.

Suspensions and oils cannot be sterilized by this method due to their heavy load of particulate matters and viscosity.

Practical 4: Media formulation for bacteria

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
- Effluent contamination

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 will be the carbon source such as carbohydrates, lipids and proteins.

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.

Organisms that cannot grow in artificial culture medium are known as **obligate parasites**. *Mycobacterium leprae, rickettsias, Chlamydias,* and *Treponema pallidum* are obligate parasites. 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 where as 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/ Basic 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
- 7g 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. B**lood 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) and Salt Milk Agar used to recover *S.aureus* contains 10% NaCl. Ingridients 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.

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

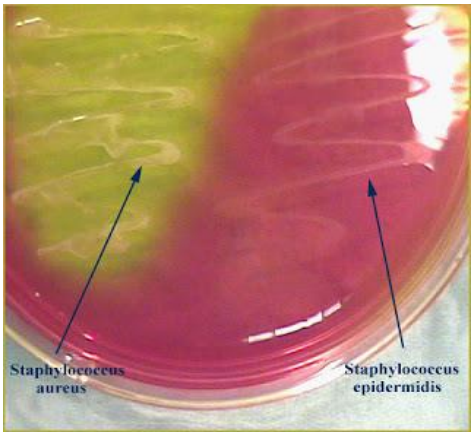
MacConkey's Agar used for **Enterobacteriaceae** members contains bile salt that inhibits most gram positive bacteria.
Composition of MacConkey agar is given below;

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/ indicator medium: differential appearance:

Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony colour. 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)
  Mac Conkey agar (lactose fermenters, pink colonies **(E-coli)** whereas non- lactose fermenter produces pale or colorless colonies.

Practical 5: Initiation of bacterial culture in a shaking flask

Introduction

Luria broth (LB) is a nutrient-rich media commonly used to culture bacteria in the lab. However, a liquid culture is capable of supporting a higher density of bacteria and is used to grow up sufficient numbers of bacteria necessary for an experimental use. LB is the standard medium used

to grow bacteria, principally Escherichia coli. Also known as Luria broth, or Luria-Bertani medium or Lennox broth. Bertani was the inventor of LB and was a student with Max Delbruck at Caltech. They were investigating phage lysis of E. Coli. The following protocol is for inoculating an overnight culture of liquid LB with bacteria.

Protocol:

1. To prepare 100 ml of LB, weigh out the following into a Adjust the pH to 5.5 with 0.1 N phosphoric acid.
    - 1 g NaCl
    - 1 g Tryptone
    - 0.5 Yeast Extract

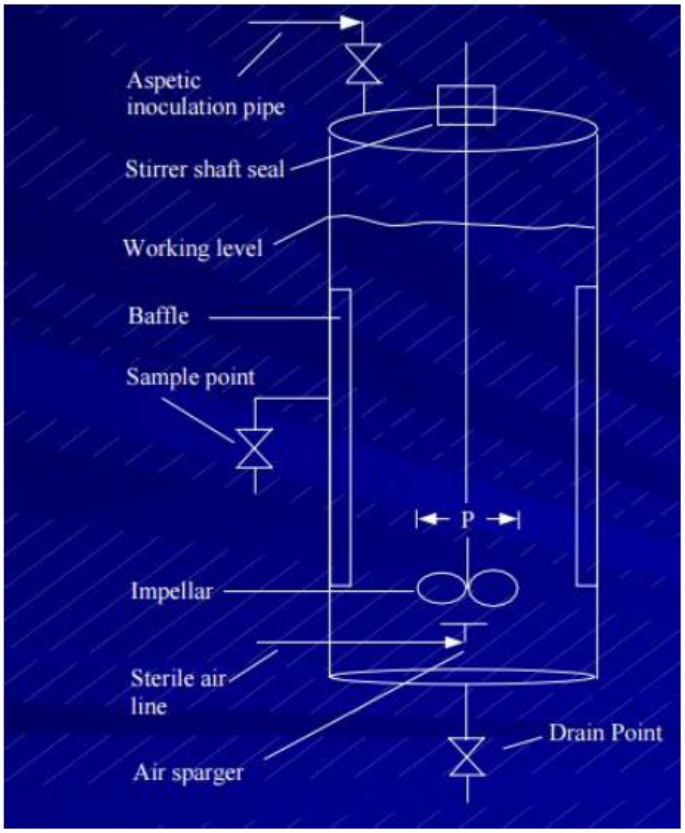
2. Make up the volume to 100 ml with distilled water.
3. Transfer medium to 250 ml Erlenmeyer flask and cotton plug it.
4. Sterilize by autoclaving at 15 psi, 121°C for 15 minutes.

Practical 6: Initiation of bacterial culture in a Fermenter

Fermentation is a metabolic process that converts sugar to acids and gases, or alcohol. It occurs in yeast and bacteria, and also in oxygen-starved muscle cells, as in the case of lactic acid fermentation. Fermentation is also used more broadly to refer to the bulk growth of microorganisms on a growth medium, often with the goal of producing a specific chemical product. French microbiologist Louis Pasteur is often remembered for his insights into fermentation and its microbial causes. The science of fermentation is known as zymology.

Construction of fermentor

A fermentor is constructed to supply filtered oxygen through the spargers located at the bottom of the fermentor. The agitator is used to keep the mixture of cells and growth media inside the fermentors relatively homogeneous. It also increases oxygen mass transfer by decreasing the size of the oxygen bubbles. The fermentor is operated at a constant growth temperature to achieve the required growth rate. Since cells liberate heat during growth, a constant temperature is maintained using either cooling jackets surrounding the fermentors or by coils inside fermentor or a combination of both. In addition, the cells secrete acids as they metaboliz , which decrease the pH level within the fermentor. As a result, a base is usually added to the fermentor whenever the pH drops below its optimum value.



Batch Fermentation

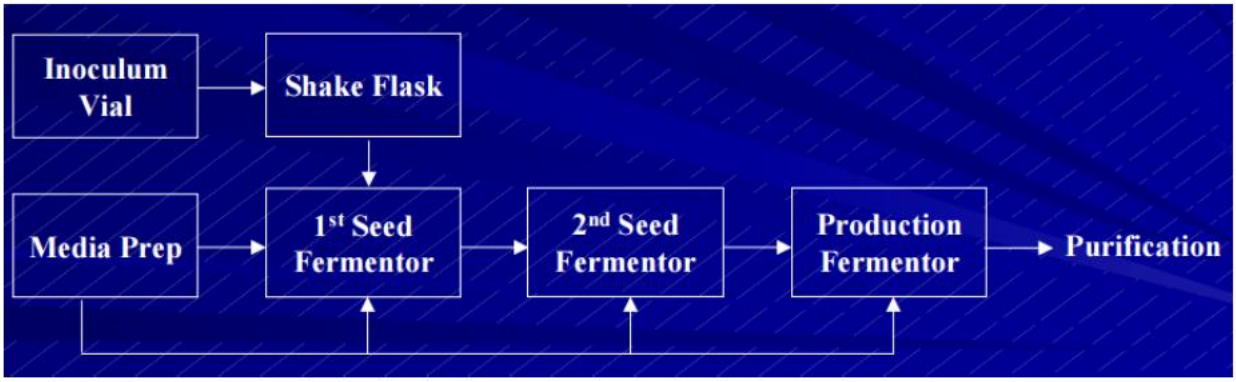
Batch Fermentation involves the process in which microbial cells grow in a batch. The Stages of Batch Fermentation

- Shake Flask
- Seed Fermentor
- Production Fermentor

An example, of a fermentation process is represented in the block flow diagram shown below.

Main Steps of Batch fermentation

1. Inoculate the LB medium with pure culture. After inoculation flask was placed in a shaking incubator at 37 °C, so the cells can grow and reproduce.
2. The cells are grown to a particular density near the end their exponential phase and used to inoculate a small fermentor known as known as a seed fermentor.
3. After the cells reach the required optical density in the seed fermentor, the cells can either be used to inoculate several used several increasingly larger seed fermentors until the required volume and density is reached, or the cells can be transferred directly to the production fermentor to where they will eventually synthesize the co - protein.
4. After achieving specific level in seed fermentation, cells are transferred in production fermentor. In production fermentor cells are grown to a particular density. The density in which they are grown depends upon the desired product being growth or non - growth associated.
5. At exponential phase, a chemical is added that induces the cells to begin over-expressing the gene responsible for the recombinant protein. Over expression leads to depletion of nutrients eventually cause the cells to enter their stationary growth phase. At this point, the cells are no longer capable of producing appreciable amounts of the desired protein and the fermentation is ended.



Practical 7: Growth Kinetics

Objectives: To establish growth curves for an unknown bacterial species and observe the different phases of growth.

Introduction:

Bacteria normally reproduce by binary fission, forming two equal-sized progeny cells and thus doubling their number with each division. This type of cell division is called exponential; i.e., one cell divides to form two, each of these cells divides so that four cells are obtained, and so forth in a geometric progression. The unit of microbial growth is "generation time", which is the time required to achieve a doubling of the population size and is designated as tgen. Generation time can be estimated by determining cell numbers during the period of active cell division and is expressed mathematically as:

$$tgen = t \ln 2 / \ln Xt - \ln X0 = t \log 2 / \log Xt - \log X0$$

In the above equations, t is the elapsed time during which growth is measured, and Xo and Xt are the number of bacteria at times zero and t, respectively. The presence of 2 in the numerator of the equations is due to the fact that when doubling of the population number occurs, the quantity Xt/Xo (called the doubling constant) will be equal to 2. The generation time of many bacteria is usually several hours. However, under optimum conditions, E. coli has a generation time of about 20 minutes. Thus it can be calculated that a single E. coli cell will produce about a thousand progeny in 3.3 hours and over a million in 6.6 hours. If the generation time and the elapsed time are known, then the number of generations can be estimated as follows:

$$\text{Number of generations} = t / tgen$$

A bacterial population follows a characteristic growth curve which has four phases: the lag phase, the log or exponential growth phase, the stationary phase, and the death phase.

1. **Lag phase:** During this phase, there is no increase in cell number; rather, bacteria are preparing for reproduction and synthesizing DNA and various inducible enzymes needed for cell division.

2. **Log phase:** This phase, also called the exponential phase, follows the lag phase and starts with a rapid increase in bacterial number. At this stage, the logarithm of bacterial biomass increases linearly with time so that numbers of bacterial cells in a given interval of time is proportional to the biomass of bacteria present.

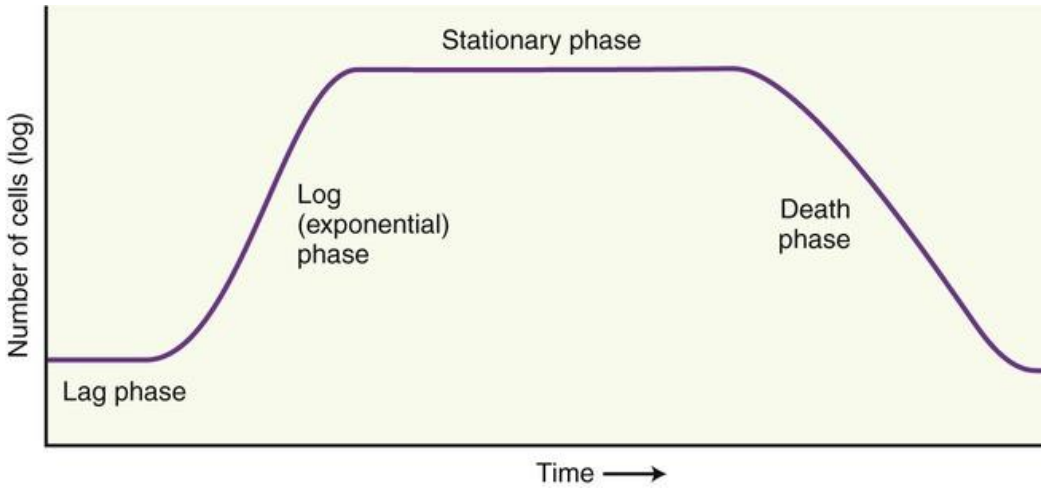
3. **Stationary phase:** The number of bacteria reaches a maximum in this phase and does not increase further (the growth rate is exactly equal to the death rate). This phase is sometimes called the plateau stage. A bacterial population may reach stationary growth when a required nutrient is exhausted, when inhibitory end products accumulate, or when physical conditions are inappropriate for growth. The duration of the stationary phase varies, with some bacteria exhibiting very long stationary phases.

4. **Death phase:** Eventually, the number of viable bacterial cells begins to decline, signaling the onset of the death phase. No further divisions occur in this phase. Death rate, in many cases, follows the same kinetics as the exponential growth.

Principle:
Turbidity, which is a measure of the growth of microorganisms, is determined via an instrument called a spectrophotometer. The basis for the use of this instrument is that less light passes through a turbid solution than through a clear solution; i.e., the more turbid a solution (more growth), the less the amount of light transmitted through the solution. Since the optical density of the culture is proportional to the cell density, measuring the turbidity of the culture solution can be used in estimating numbers of bacterial cells, if a growth curve for the conditions used has already been established. This is the most common method used to rapidly estimate bacterial numbers.

Laboratory Supplies: 10 ml culture of an unknown microorganism, 50 ml LB broth in 125-ml flasks, Spectrophotometer, Un-inoculated LB broth in small test tube for blank.

Procedure:
1. Obtain a flask of LB and transfer 5 ml of broth to a small test tube. This is your blank. Adjust the wavelength to 550 nm on the spectrophotometer and calibrate the machine to 0.0 O.D. or 100% transmittance with your blank. Take the blank out but do not discard it.
2. Inoculate your flask with 10 ml of the culture provided and swirl the flask well.
3. Work quickly to withdraw 5 ml of the sample into a clean small test tube, using a pipette.
4. Place the culture flask immediately at the assigned incubating temperature and aeration condition and record the time.
5. Measure the O.D. of your sample next. Record the 0 time absorbance. Discard the contents of your sample test tube. This test tube can be re-used for your next sample without washing.
6. At 15-minute intervals for up to 90 minutes, shake your flask vigorously and transfer 5 ml of the culture to your sample test tube.
7. Record the optical density at each time period. Use your blank each time to make sure your readings are correct and other students have not altered the machine's calibration. Your instructor will ask all groups to write their results in a table on the blackboard. Copy these results to your Results Sheet. If more than one group is doing a growth condition, get their average data.
8. Plot the optical density (y-axis) versus time (x-axis) for each growth condition on the same regular graph paper.



Experiment 8: Medium formulation (fungi)

Potato dextrose agar and potato dextrose broth are common microbiological growth media made from potato infusion, and dextrose. Potato dextrose agar (abbreviated "PDA") is the most widely used medium for growing fungi.
Potato infusion can be made by boiling 200 grams of sliced (washed but unpeeled) potatoes in ~ 1 litre of distilled water for 30 minutes and then decanting or straining the broth through cheesecloth. Distilled water is added such that the total volume of the suspension is 1 litre. PDA is also available commercially as premixed medium.

Composition (g/L) of PDA

- Potato infusion 200 g
- Dextrose 20 g
- Agar 15 g
- pH:5.5

Malt Extract Agar is used as a general purpose growth media to isolate and cultivate molds from clinical samples, as well as a wide range of environmental sources. Malt Extract Agar is designed to contain the proper formulation of carbon, protein and nutrient sources essential for yeast and mold growth. Dextrose is added to the medium to provide a carbon and energy source for fungi. Additionally, Malt Extract Agar contains digests of animal tissues (peptones) which provide a nutritious source of amino acids and nitrogenous compounds for the growth of mold. The pH is adjusted to approximately 5.5 in order to enhance the growth of fungi and to slightly inhibit bacterial growth commonly found as environmental contaminants.

Composition of Malt Extract Agar Medium

Ingredients per liter of deionized water:*

Malt Extract Agar:	
Malt Extract	20.0gm
Dextrose	20.0gm
Peptone	6.0gm
Agar	15.0gm

Procedure (Preparation of PDA)

1. Add 3.9 grams 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.

Experiment 9: Medium formulation (Yeast)

YEPD or **yeast extract peptone dextrose**, also often abbreviated as **YPD**, is a complete medium for yeast growth. It contains yeast extract, peptone and glucose or dextrose. It can be used as solid medium by including agar. The yeast extract will typically contain all the amino acids necessary for growth. YEPD is used as a growth medium to grow yeast cultures.

Composition (g/L)

Peptone 20
Yeast extract 10
Dextrose 20
pH:4-4.5

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 5.5 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.

Experiment 10: Production of enzyme (alpha amylase) by solid state fermentation.

Introduction:

Fermentation actually comes from Latin word "fervere" which means "to boil". So the word "Fermentation" is referred to physical state of boiling/bubbling.

Fermentation may be defined as "process of converting carbohydrates to alcohol or organic acids using microorganisms (yeast/bacteria) under anaerobic conditions"

Or

Fermentation is a process of mass culturing of cells for the sake of desired product e.g. acids, enzymes, gases, alcohols, hormones etc.

Types of fermentation

There are three types of fermentation on the basis of Moisture/Water (H_2O) content in medium.

1. Solid state fermentation
2. Submerged fermentation
3. Surface fermentation (Organism is allowed to grow on the surface of a liquid medium without agitation. After an appropriate incubation period the culture filtrate is separated from the cell mass and is processed to recover the desirable product)

- **Solid-State Fermentation (SSF)**

In solid-state fermentation, the microorganisms grow on a moist solid with little or no 'free' water, although capillary water may be present.

Examples of this type of fermentation are seen in mushroom cultivation, bread-making and the processing of cocca, and in the manufacture of some traditional foods, e.g. miso (soy paste), soy sauce, tempeh (soybean cake) and gari (cassava), which are now produced in large industrial operations. SSF is also called as Koji Fermentation.

Advantages of solid state fermentation

1. Cheap
2. environmental friendly
3. High yield
4. Low availability of water reduces possibilities of contamination

Disadvantages

1. Downstream processing difficult
2. difficult to scale up
3. difficult to monitor and control different parameters

Materials Required

Beaker, Stirrer, 250 ml Erlenmeyer flask, cylinder, wheat bran, $Zn\ SO_4.7H_2O$, $FeSO_4.7H_2O$, $Cu\ SO_4.7H_2O$

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 (containing mg/L: $Zn\ SO_4.7H_2O$, 6.2; $FeSO_4.7H_2O$, 6.8; $Cu\ SO_4.7H_2O$, 0.8; Distilled water, 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 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).

Experiment 11: Production of enzyme (alpha amylase) by submerged fermentation.

Introduction

In submerged fermentation, microorganisms grow in submerged state within the fermentation media. Submerged fermentation may use a dissolved substrate, e.g. sugar solution, or a solid substrate, suspended in a large amount of water to form a slurry. Organism is grown in a liquid medium which is vigorously aerated & agitated.

It is used for pickling vegetables, brewing beer and producing wine and soy sauce and production of enzymes.

Advantages

1. ensure proper agitation and aeration
2. Downstream processing easy
3. Possibility to automate many operations
4. Uniform distribution of nutrient and waste products.
5. Various parameters can be monitored or controlled easily (by periodic sampling of broth) and controlled if necessary by addition of further nutrients and reagents.

Disadvantages

1. costly
2. low yield as compared to solid state fermentation

Materials Required

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

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; $FeCl_3$, agar, 15; 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 conidial suspension to flask.
5. Incubate the flask at 37°C for 48 h.
6. After fixed period of incubation, centrifuge the contents of the flask at 6000 rpm for 15 minutes.
7. Use the supernatant for the estimation of enzyme (alpha amylase).

Experiment No 12: Partial purification of enzyme by ammonium sulfate precipitation.

Introduction

Ammonium sulfate precipitation is one of the most commonly used methods for large and laboratory scale protein purification and fractionation that can be used to separate proteins by altering their solubility in the presence of a high salt concentration.

Ammonium sulfate is an inorganic salt with a high solubility that disassociates into ammonium (NH_4^+) and sulfate (SO_4^{2-}) in aqueous solutions.[1] Ammonium sulfate is especially useful as a precipitant because it is highly soluble, stabilizes protein structure, has a relatively low density, is readily available, and is relatively inexpensive.

The solubility of proteins varies according to the ionic strength of the solution, thus according to the salt concentration. As the salt concentration is increased, the solubility of the protein begins to decrease. At a sufficiently high ionic strength, the protein will precipitate out of the solution, an effect termed "salting out".

Proteins differ markedly in their solubilities at high ionic strength, therefore, "salting out" is a very useful procedure to assist in the purification of the desired protein. In solution, proteins form hydrogen bonds with water molecules through their exposed polar and ionic groups. When high concentrations of small, highly charged ions such as ammonium sulfate are added, these groups compete with the proteins to bind to the water molecules. This removes the water molecules from the protein and decreases its solubility, resulting in precipitation. Critical factors that affect the concentration at which a particular protein will precipitate include: the number and position of polar groups, molecular weight of the protein, pH of the solution, and temperature at which the precipitation is performed.

Ammonium sulfate precipitation is a useful technique as an initial step in protein purification because it enables quick, bulk precipitation of cellular proteins.[5] It is also often employed during the later stages of purification to concentrate protein from dilute solution following procedures such as gel filtration.

Procedure

1. Use a graduated cylinder to measure the volume of the fermented broth to be concentrated. Pour the solution into a beaker with a capacity approximately twice the measured volume of the fermented broth. Place the beaker on ice.

2. In the ammonium sulfate tables, find the amount of solid ammonium sulfate required to give the percentage of saturation (10%). Weigh out the necessary amount of ammonium sulfate. If the ammonium sulfate contains lumps, use a mortar and pestle to crush the lumps to a smooth powder.

3. Place the beaker containing the chilled fermented broth (and a large magnetic stir bar) on a large stir plate at 4°C.

4. Sprinkle small batches of the solid ammonium sulfate onto the surface of the stirring fermented broth so that it takes ~30 minutes to add all of the ammonium sulfate. Use a slow rate of stirring to avoid foaming. If solid ammonium sulfate accumulates at the bottom of the beaker, stop the addition and allow the ammonium sulfate to dissolve before adding more solid.

5. After the addition of ammonium sulfate is complete, stir the mixture for another 60 minutes

6. Transfer the mixture to centrifuge tubes and centrifuge the tubes at 10,000 *g* for 15 minutes at 4°C.

7. Resuspend the protein pellet (if any) in the minimum volume of appropriate buffer (e.g. 20 mM phosphate buffer)

8. Assay the protein solution for the presence of the target protein by enzyme assay.

9. Transfer the supernatant to another beaker. Measure the volume of the supernatant and add the amount of solid ammonium sulfate required to give the percentage of saturation (20%).

10. Repeat steps 3-8.

11. Then keep on increasing the percentage of saturation by 10% till the pellet contains the desired protein.

12. If necessary, remove any residual ammonium sulfate from the protein solution by dialysis, ultrafiltration, or chromatographic desalting.

Initial concentration of ammonium sulphate, (% saturation)	Final concentration of ammonium sulphate, % saturation																
	10	20	25	30	33	35	40	45	50	55	60	65	70	75	80	90	100
	Grams solid ammonium sulphate to be added to 1 L of solution																
0	56	114	144	176	196	209	243	277	313	351	390	430	472	516	561	662	767
10		57	86	118	137	150	183	216	251	288	326	365	406	449	494	592	694
20			29	59	78	91	123	155	189	225	262	300	340	382	424	520	619
25				30	49	61	93	125	158	193	230	267	307	348	390	485	583
30					19	30	62	94	127	162	198	235	273	314	356	449	546
33						12	43	74	107	142	177	214	252	292	333	426	522
35							31	63	94	129	164	200	238	278	319	411	506
40								31	63	97	132	168	205	245	285	375	469
45									32	65	99	134	171	210	250	339	431
50										33	66	101	137	176	214	302	392
55											33	67	103	141	179	264	353
60												34	69	105	143	227	314
65													34	70	107	190	275
70														35	72	153	237
75															36	115	198
80																77	157
90																	79