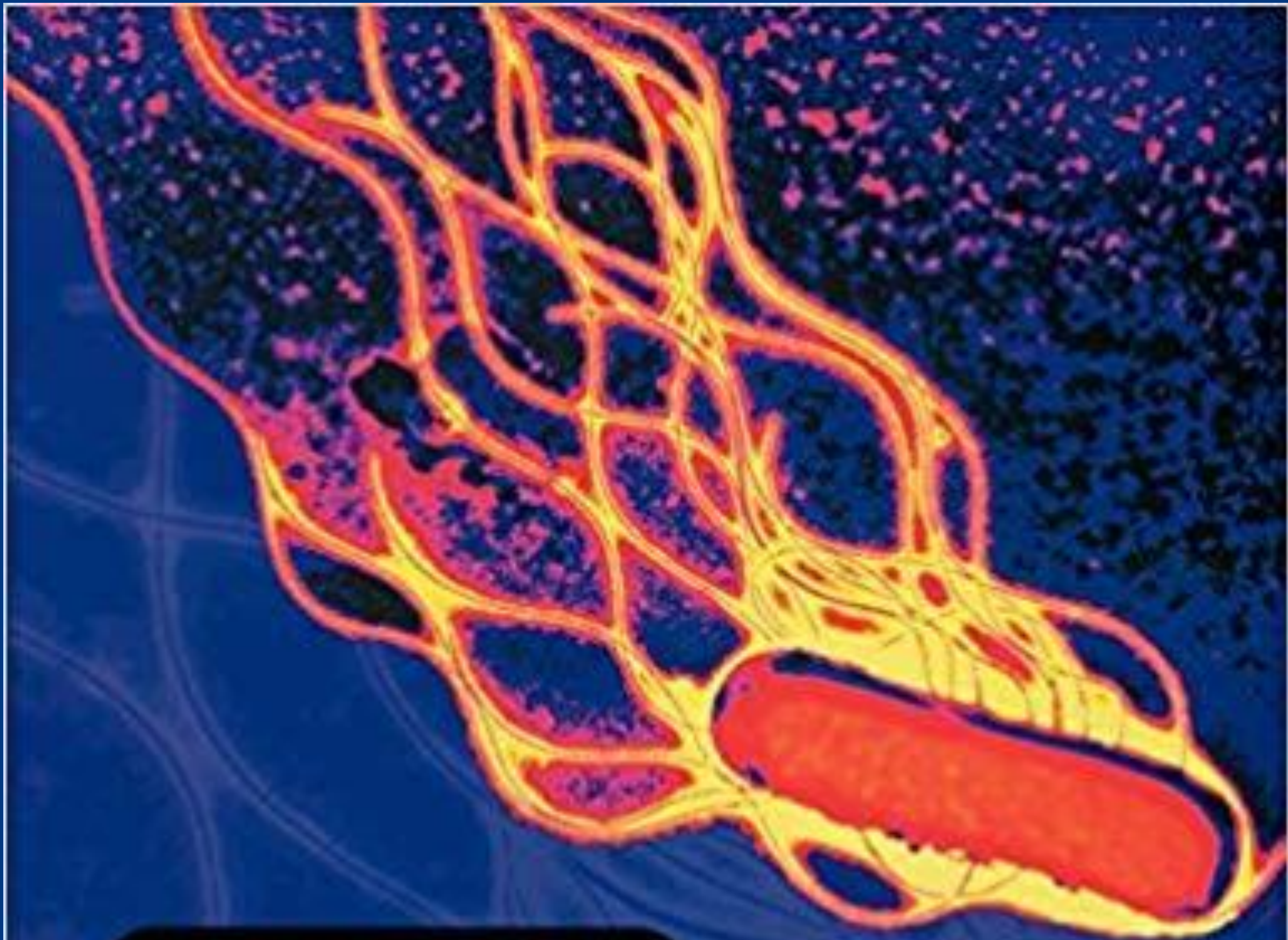


INDUSTRIAL

Microbiology

What is Industrial Microbiology?

Industrial microbiology is the commercial exploitation of microorganisms to produce valuable economic, environmental and socially important products, or to carry out important chemical transformations.

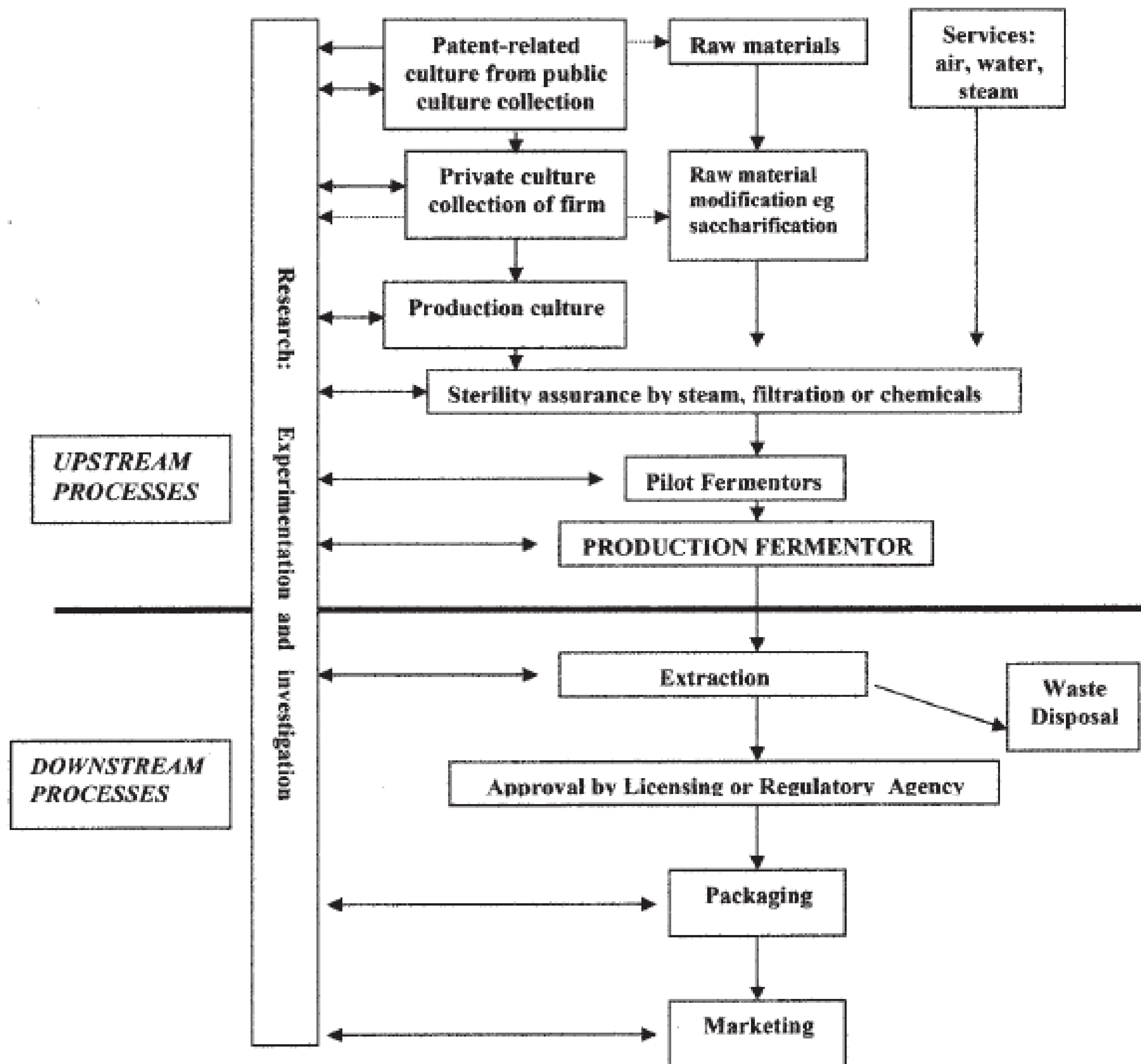
Industrial microbiology is study of the large-scale and profit motivated production of microorganisms or their products for direct use, or as inputs in the manufacture of other goods.

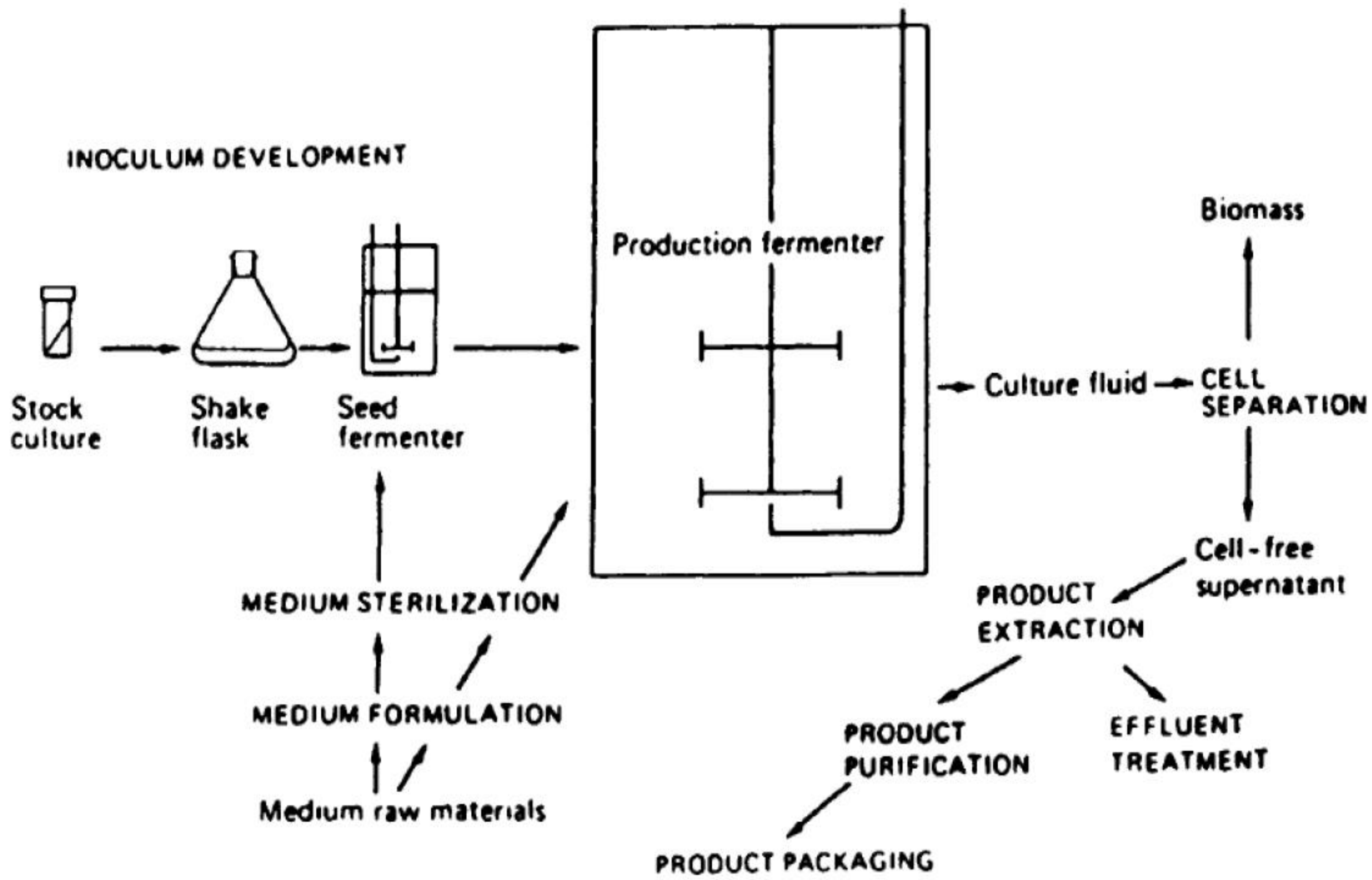
Example

- Yeasts may be produced for direct consumption as food for humans or as animal feed, or for use in bread-making;
- Their product, ethanol, may also be consumed in the form of alcoholic beverages, or used in the manufacture of perfumes, pharmaceuticals, etc.

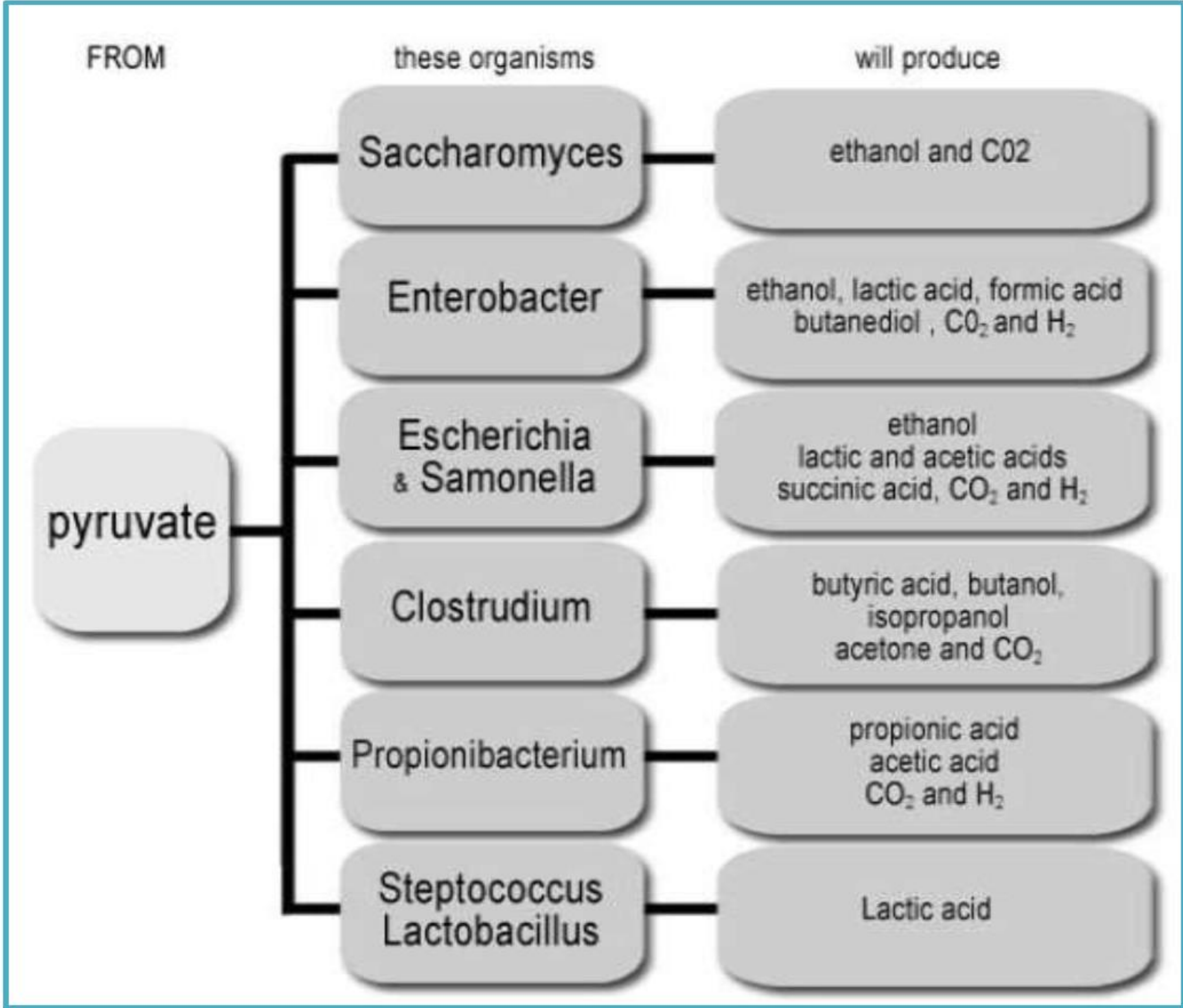
TABLE 19.3 A selected list of important microorganisms and their products

Microorganism	*Product*
Algae	
Chlorella sorokiniana	Single-cell protein
Spirulina maxima	Single-cell protein
Bacteria	
Acetobacter aceti	Acetic acid
Acetobacter woodii	Acetic acid
Bacillus subtilis	Bacitracin
B. brevis	Gramicidin
B. thuringiensis	Endotoxin
Clostridium aceticum	Acetic acid
Methylophilus methylotrophus	Glutamic acid
Pseudomonas denitrificans	Vitamin B_{12}
Actinomycetes	
Streptomyces aureofaciens	Tetracycline
S. griseus	Streptomycin
S. tradiae	Neomycin
Nocardia mediterranei	Rifamycin
Micromonospora purpurea	Gentamycin
Fungi	
Aspergillus niger	Citric acid
A. oryzae	Amylase, cellulase, single-cell protein
Candida lipolytica	Lipase
C. utilis	Single–cell protein
Penicillium chrysogenum	Penicillin
Saccharomyces cerevisiae	Ethanol, wine, single-cell protein
S. lipolytica	Citric acid, single-cell protein
Rhizopus nigricans	Steroids
Gibberella fujikuroi	Gibberellin
Trichoderma viride	Cellulase


Patent-related culture from public culture collection
Raw materials
Services: air, water, steam
Private culture collection of firm
Raw material modification eg saccharification
Production culture
Research: Experimentation and investigation
Sterility assurance by steam, filtration or chemicals
UPSTREAM PROCESSES
Pilot Fermentors
PRODUCTION FERMENTOR
Extraction
Waste Disposal
DOWNSTREAM PROCESSES
Approval by Licensing or Regulatory Agency
Packaging
Marketing



INOCULUM DEVELOPMENT
Production fermenter
Biomass
Stock culture
Shake flask
Seed fermenter
Culture fluid
CELL SEPARATION
Cell-free supernatant
PRODUCT EXTRACTION
MEDIUM STERILIZATION
MEDIUM FORMULATION
Medium raw materials
PRODUCT PURIFICATION
EFFLUENT TREATMENT
PRODUCT PACKAGING


FROM
these organisms
will produce
pyruvate
Saccharomyces
ethanol and C02
Enterobacter
ethanol, lactic acid, formic acid
butanediol , $C0_2$ and H_2
Escherichia
& Samonella
ethanol
lactic and acetic acids
succinic acid, CO_2 and H_2
Clostrudium
butyric acid, butanol,
isopropanol
acetone and CO_2
Propionibacterium
propionic acid
acetic acid
CO_2 and H_2
Steptococcus
Lactobacillus
Lactic acid

Requirements for industrially useful microorganisms

- To be used in industrial microbiology microorganism must be
  - Grow in simple media: preferably not require growth factors
  - Grow vigorously and rapidly
  - Produce the desired product in short time possible
  - Its end products should not include toxic and other undesirable materials
  - The organism should have a reasonable genetic, and hence physiological stability.
  - The organism should lend itself to a suitable method of product harvest at the end of the fermentation.

- Wherever possible, organisms which have physiological requirements which protect them against competition from contaminants should be used.
- The organism should be reasonably resistant to predators
- the organism should not be too highly demanding of oxygen
- organism should be easily amenable to genetic manipulation to enable the establishment of strains with more acceptable properties.

Fermentation

- The basic principle involved in the industrial fermentation technology is that organisms are grown under suitable conditions, by providing raw materials meeting all the necessary requirements such as carbon, nitrogen, salts, trace elements and vitamins.

- The end products formed as a result of their metabolism during their life span are released into the media, which are extracted for use by human being and that have a high commercial value.

What is fermentation techniques (1)?

Techniques for large-scale production of microbial products. It must both provide an optimum environment for the microbial synthesis of the desired product and be economically feasible on a large scale. They can be divided into surface (emersion) and submersion techniques. The latter may be run in batch, fed batch, continuous reactors

In the surface techniques, the microorganisms are cultivated on the surface of a liquid or solid substrate. These techniques are very complicated and rarely used in industry

In the submersion processes, the microorganisms grow in a liquid medium. Except in traditional beer and wine fermentation, the medium is held in fermenters and stirred to obtain a homogeneous distribution of cells and medium. Most processes are aerobic, and for these the medium must be vigorously aerated. All important industrial processes (production of biomass and protein, antibiotics, enzymes and sewage treatment) are carried out by submersion processes.

Fermentation

- **Definition**
  - i. *by biochemist*
    - i. anaerobic process that generate energy by the breakdown of organic compounds.
    - ii. Any process that generate bacterial metabolites as end products: lactic acid, enzymes, ethanol, butanol, and acetone.
  - ii. *by indusrial users*
    - i. Any aerobic process that produces microorganisms (biomass)as the end product.
    - ii. Biotransformation--transformation of a compound by microbial cells
- Industrial biotechnology, application of modern biotechnology for industrial production, is always referring to Fermentation technology.
  - Less waste generation
  - Reduced energy consumption
- Fermentation biotechnology involves partnership between
  - **Molecular biologists** ; responsible for isolating, characterising, modifying and creating effectively expression of industry desirable genes
  - **Biochemical engineers** ; to ensure that the GE of microorganisms can be grown in large quantities under the conditions that give optimal product yeild.

FERMENTER(bioreactor)

- Closed container with adequate arrangement for aeration, agitation, temperature and pH control, and drain or overflow vent to remove the waste biomass of cultured microorganisms along-with their products.
- Is a device in which a substrate of low value is utilized by living cells or enzymes to generate a product of higher value.
- Extensively used for food processing, fermentation, waste treatment, etc.

BIOREACTOR

❑ A bioreactor should provide for the following:

- Agitation (for mixing of cells and medium),
- Aeration (aerobic fermenters); for O2 supply,
- Regulation of factors like temperature, pH, pressure, aeration, nutrient feeding, liquid level etc.,
- Sterilization and maintenance of sterility, and
- Withdrawal of cells/medium (for continuous fermenters).

✓ Modern fermenters are usually integrated with computers for efficient process monitoring, data acquisition, etc.

Bioreactor

How a bioreactor differs from a chemical reactor ?

Both are agitated tanks

Bioreactor should be capable of being operated aseptically for number of days

Adequate aeration and agitation should be provided to meet the metabolic requirements which will vary from time to time

What is a Fermenter?

- Vessel or tank in which whole cells or cell-free enzymes transform raw materials into biochemical products and/or less undesirable by-products
- Also termed a Bioreactor

- The basic function of a fermenter is to provide a suitable environment in which an organism can efficiently produce a target product

SIZE OF FERMENTERS(BIOREACTOR):

- The size of fermenters ranges from 1-2-liter laboratory fermenters to 5,00,000 liters or, occasionally, even more, fermenters of up to 1.2 million liters have been used.
- The size of the fermenter used depends on the process and how it is operated.

BIOREACTOR

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- Withdrawal of cells/medium (for continuous fermenters).

✓Modern fermenters are usually integrated with computers for efficient process monitoring, data acquisition, etc.

Bioreactor	Fermenter
• A bioreactor can be defined as an apparatus, such as a large fermentation chamber, for growing organisms such as bacteria or yeast that are used in the biotechnological manufacture of substances such as pharmaceuticals, antibodies, or vaccines, or for the bioconversion of organic waste.	• However a fermenter can be defined as an apparatus that maintains optimal conditions for the growth of microorganisms, used in large-scale fermentation and in the commercial production of antibiotics and hormones

BIOREACTOR VERSUS FERMENTOR

BIOREACTOR	FERMENTOR
An apparatus in which a biological reaction or process is carried out, especially on an industrial scale	A container in which fermentation takes place
Allows any type of biochemical reactions to occur	Only facilitate fermentation
A vessel that facilitates a biochemical reaction	A type of bioreactor
Various types of substrates can be used based on the desired reaction	Glucose or glucose-containing compounds are used
May use microorganisms or biochemically active substances such as enzymes or catalysts	Always uses microorganisms to carry out the reaction
Can use mammalian or insect cell populations	Use fungal or bacterial cell populations
Microorganisms are introduced	Microorganisms in the air are used
May use either aerobic or anaerobic conditions	Use anaerobic conditions
Volume can be up to several liters	Volume can be up to 2L
A preferable agitation RPM has to be maintained due to the presence of cells without cell walls	A considerable agitation RPM can be used since both bacteria and fungi have cell walls
Doubling time is long (14, 17 or 24 hours)	Doubling time is 20 mins
Can either be used to produce a cell mass or a particular metabolite	Used to produce a metabolite
Can produce secondary metabolites	Can only produce primary metabolites
Used in the production of medicines, pharmaceutical liquids, antibodies or vaccines	Used to produce lactic acid or ethanol
Tend to be infected by viruses	Generally not infected by viruses
Can be packed bed, fluidized bed, IVFR or Air lift bioreactor	Can be batch, fed batch or continuous

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DESIGN OF FERMENTER

- A fermentation process requires a fermenter for successful production .
- Fermentor is the large vessel containing considerable quantities of nutrient media by maintaining favourable conditions.
- The design and nature of the fermentor varies depending upon the type of fermentation carried out. Invariably all the fermentors provide the following facilities for the process such as
  - contamination free environment,
  - specific temperature maintenance,
  - maintenance of agitation and aeration, pH control,
  - monitoring Dissolved Oxygen (DO),
  - ports for nutrient and reagent feeding (antifoam agents, alkali or acid),
  - ports for inoculation and sampling,
  - provide all aseptic conditions at the time of sample withdrawal and addition of innoculum
  - complete removal of broth from the tank and should be easy to clean
  - It should be designed in such away that it consumes less power, have less evaporation, can be used for long periods of operation

Fermenter: General Functions

What it should be capable of:

- Biomass concentration must remain high
- Maintain sterile conditions
- Efficient power consumption
- Effective agitation
- Heat removal
- Sampling facilities

Common features of typical fermenter:

1. They should be strong enough to withstand the pressure exerted by large volume of the medium.
2. The materials used for the construction of fermenter should not be corroded by the fermentation product and it should not yield toxic ion to the medium.
3. The fermenter should have provision for the control and prevention of the growth of contaminating microorganisms because industrial fermentation requires pure culture.
4. If aerobic organisms are used in the process, there should be provision for rapid incorporation of sterile air into the medium so that the oxygen is immediately dissolved in the medium and available to the microorganisms.
5. The Carbon dioxide produced by the microorganisms should be removed from the medium.

Common features of typical fermenter:

6. Stirring is necessary to mix the organisms with the medium and to make nutrients and oxygen available to individual microbe.

7. The fermenter should provide provision for the addition of antifoaming agents intermittently depending on the foaming status of the medium.

8. Thermostatic system should be available to maintain constant temperature in the fermenter.

9. There should be provision for aseptic withdrawal of culture during fermentation and also for the aseptic introduction of inoculum at the starting of the fermentation process.

10. A system should be available for detection of pH of the culture medium and also for its adjustment.

Fermenter construction

- All materials must be corrosion resistant to prevent trace metal contamination of the process
- Materials must be non-toxic so that slight dissolution of the material or components do not inhibit culture growth
- Materials of the fermenter must withstand repeated sterilization with high pressure steam
- Fermenter stirrer system and entry ports be sufficiently robust not to be deformed under mechanical stress
- Visual inspection of the medium and culture is advantageous, transparent materials should be used

CONSTRUCTION OF FERMENTERS:

- Large-scale industrial fermenters are almost always constructed of stainless steel.
- A fermenter is a large cylinder closed at the top and the bottom and various pipes and valves are fitted into it.

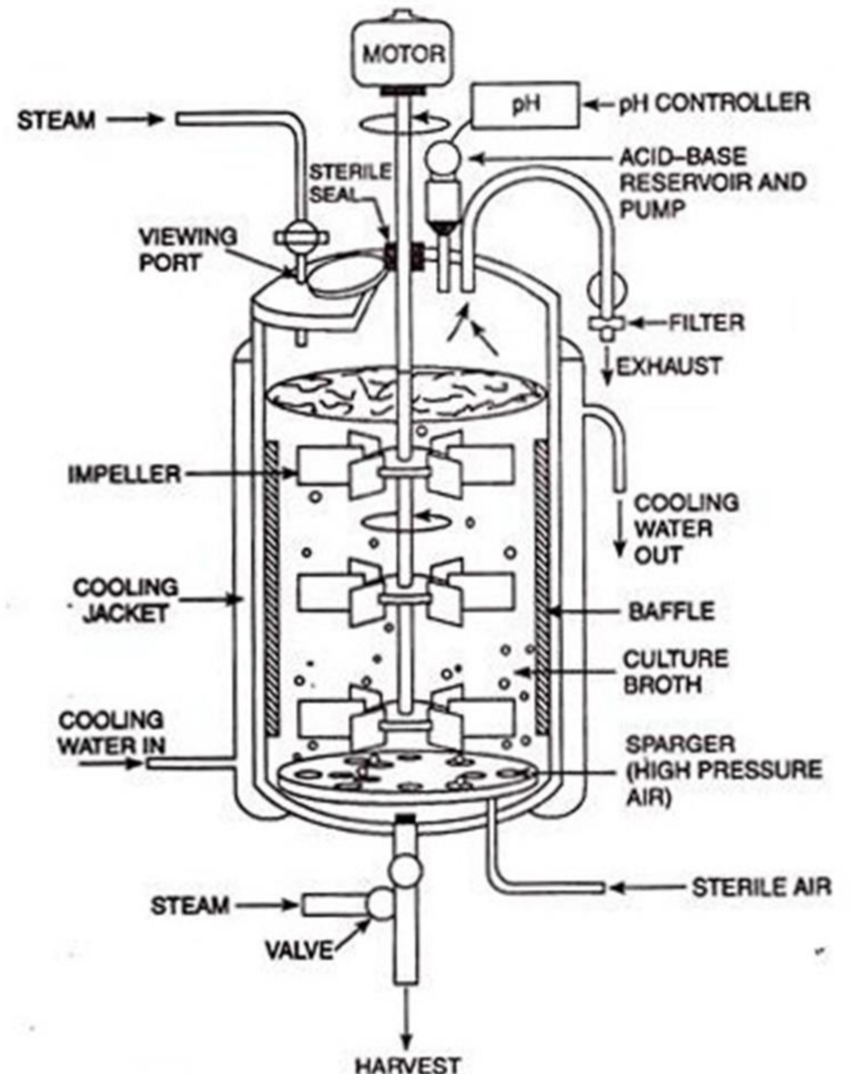


FIG. 39.1. An industrial aerobic fermentor (internal view)

Sr.	Parts of fermenter	Function
1	Impellor (agitator)	To stir the media continuously and hence prevent cells from settling down, and distribute oxygen throughout the medium
2	Sparger (Aerator)	Introduce sterile oxygen to the media in case of aerobic fermentation process
3	Baffles (vortex breaker)	Disrupt vortex and provide better mixing
4	Inlet Air filter	Filter air before it enter the fermenter
5	Exhaust Air filter	Trap and prevent contaminants from escaping
6	Rotameter	Measure flow rate of Air or liquid
7	Pressure gauge	Measure pressure inside the fermenter
8	Temperature probe	Measure and monitor change in temperature of the medium during the process
9	Cooling Jacket	To maintain the temperature of the medium throughout the process
10	pH probe	Measure and monitor pH of the medium
11	Dissolve Oxygen Probe	Measure dissolve oxygen in the fermenter
12	Level probe	Measure the level of medium
13	Foam probe	Detect the presence of the foam
14	Acid	Maintain the required pH of the medium by neutralizing the basic environment
15	Base	Maintain the required pH of the medium by neutralizing the acidic environment
16	Antifoam	Breakdown and prevent foams
17	Sampling pint	To obtain samples during the process
18	Valves	Regulation and control the flow liquids and gases
19	Control panel	Monitor over all parameters

CONSTRUCTION OF FERMENTERS(BIOREACTOR):

- Since most industrial fermentation process is aerobic, the construction of a typical aerobic fermenter is the following:

I. Cooling Jacket:

- The fermenter is fitted externally with a cooling jacket through which steam (for sterilization) or cooling water (for cooling) is run.
- Cooling jacket is necessary because sterilization of the nutrient medium and removal of the heat generated are obligatory for successful completion of the fermentation in the fermenter.

II. Aeration system:

- Critical part of a fermenter.
- In a fermenter with a high microbial population density, there is a tremendous oxygen demand by the culture, but oxygen being poorly soluble in water hardly transfers rapidly throughout the growth medium.
- Two separate aeration devices are used to ensure proper aeration in fermenter.

i. Sparger (series of holes in a metal ring)

ii. Impeller(also called agitator) → device necessary for stirring of the fermenter.

- The stirring accomplishes two things:

i. It mixes the gas bubbles through the liquid culture medium and
ii. It mixes the microbial cells through the liquid culture medium. In this way, the stirring ensures uniform access of microbial cells to the nutrients.

III. Baffles:

- The baffles are metal strips normally incorporated into fermenters of all sizes to prevent a vortex and to improve aeration efficiency.

IV. Controlling Devices for Environmental Factors

- Environmental factors that are frequently controlled includes temperature, oxygen concentration, pH, cells mass, levels of key nutrients, and product concentration.

Aeration is to provide microorganism in submerged culture with sufficient oxygen for metabolic requirements.

Agitation ensures that a uniform suspension of microbial cells is achieved in a homogenous nutrient medium.

Aeration and agitation depends on fermentation.

- Fine bubble aerator without agitation: Advantage of lower equipment and power costs,
- Agitation may be dispensed with only when aeration provides sufficient agitation.
- E.g. in processes when broth of low viscosity and low total solids.
- Mechanical agitation is required for fungal and actinomycetes fermentation.

Structural components of aeration and agitation

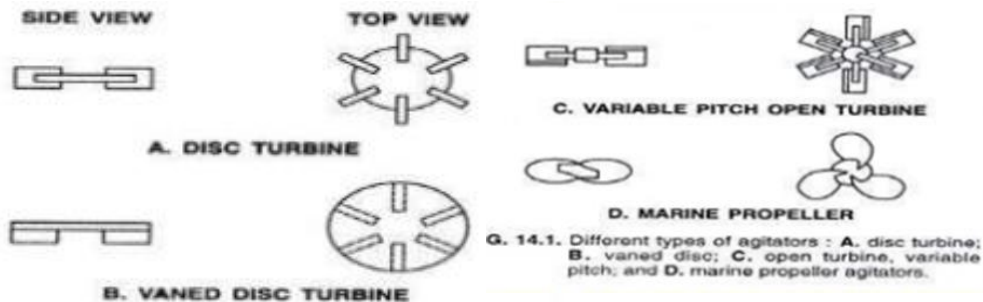
- 1. The agitator (Impeller)
- 2. Stirrer glands and bearings
- 3. Baffles
- 4. The aeration system (Sparger)

Importance of agitation

- 1. To increase the rate of oxygen transfer from the air bubble to the liquid medium.
- 2. To increase the rate of oxygen and nutrients transfer from the medium to cells.
- 3. To prevent formation of clumps of cells, aggregates of mycelium.
- 4. To increase the rate of transfer of product of metabolism from cell to medium.
- 5. To increase the rate or efficiency of heat transfer between the medium and the cooling surfaces of the fermenters.

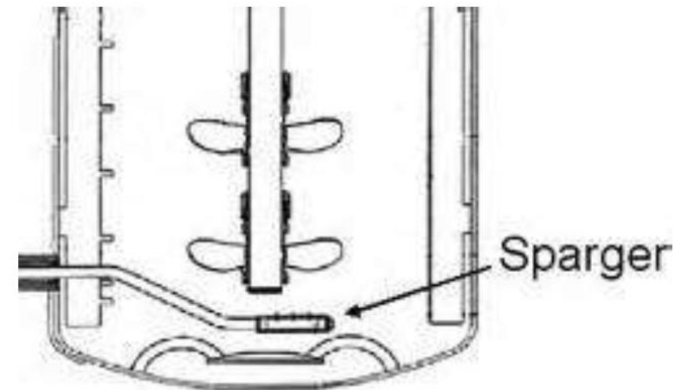
The agitator

- Agitator is required to mix the following objectives:
- bulk fulid and gas-phase mixing, air dispersion, oxygen transfer, hear transfer, suspension of solid particles and maintaining uniform environment.
- Types: Disc turbines, vaned dics, open turbines of variable pitch and propellers.



The aeration system (Sparger)

- A sparger is a device for introducing air into the liquid in a fermenter.
- Types:
- Porous sparger
- The orifice sparger (a perforated pipe)
- The nozzle sparger (an open or partially closed pipe)



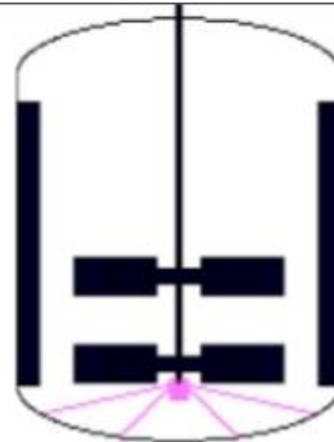
Baffles

- Four baffles to prevent vortex and to improve aeration efficiency.
- Vessel with3dm3 – six to eight baffles.
- They are metal strips, one-tenth of vessel dia. And attached radially to the wall.
- Increased agitation with wider baffles; drop in agitation with narrower baffles.

3. Baffles:

Baffles are metal strips roughly one-tenth of the vessel diameter and attached radially to the fermenter wall.They are normally used in fermenters having agitators to prevent vortex formation and to improve aeration efficiency

Usually, four baffles are used, but larger fermenters may have 6 or 8 baffles. Extra cooling coils may be attached to baffles to improve cooling. Further, the baffles may be installed in such a way that a gap exists between the baffles and the fermenter wall. This would lead to a scouring action around and behind the baffles, which would minimise microbial growth on the baffles and the fermenter wall.



TYPES AND CLASSIFICATIONS OF BIOREACTORS

Bioreactors are generally classified into two broad groups;

1. SUSPENDED GROWTH BIOREACTORS;

The reactors use microbial metabolism under aerobic, anaerobic, or sequential anaerobic/aerobic conditions to biosorb organic compounds and biodegrade them to innocuous residuals. The microbial activity in the systems produces biomass that is removed by gravity sedimentation, with a portion of the settled biomass recycled to maintain a desired mixed liquor suspended solids concentration in the bioreactor. E.g Batch reactors, CSTR'S, Plug-flow reactors etc

2. BIOFILM BIOREACTORS:

In biofilm reactors most of the microorganisms are attached to a surface, and in this manner kept within the reactor. Biofilm is also used regularly for wastewater treatment, and the bacteria can either absorb or break down toxic substances in the water. The different kinds of biofilm reactors include membrane, fluidized bed, packed bed, airlift, and upflow anaerobic sludge blanket reactors.

TYPES OF FERMENTER:

Following are the types of fermenter

- Airlift Fermenter:
- Continuous Stirred Tank Bioreactors:
- Photo-Bioreactors:
- Bubble Column Fermenter:
- Fluidized Bed Bioreactors:
- Packed Bed Bioreactors:

Airlift Fermenter:

- In airlift fermenter the liquid culture volume of the vessel is divided into two interconnected zones by means of a baffle or draft tube.
- Only one of the two zones is sparged with air or other gas and this sparged zone is known as the riser.
- The other zone that receives no gas is called down-comer.
- The bulk density of the gas-liquid dispersion in the gas-sparged riser tends to be lower than the bulk density in the down-comer.
- consequently the dispersion flows up in the riser zone and down-flow occurs in the down-comer.

- Airlift fermenters are highly energy-efficient.
- They are often used in large-scale manufacture of biopharmaceutical proteins obtained from fragile animal cells.
- Heat and mass transfer capabilities of airlift reactors are at least as good as those of other systems.
- Airlift reactors are more effective in suspending solids than are bubble column fermenters.
- All performance characteristics of airlift -fermenter are related ultimately to the gas injection rate and the resulting rate of liquid circulation.

- The rate of liquid circulation increases with the square root of the height of the airlift device.
- Because the liquid circulation is driven by the gas hold-up difference between the riser and the down-comer.
- circulation is enhanced if there is little or no gas in the down-comer.
- All the gas in the down-comer comes from being entrained in with the liquid as it flows into the down-comer from the riser near the top of the reactor.

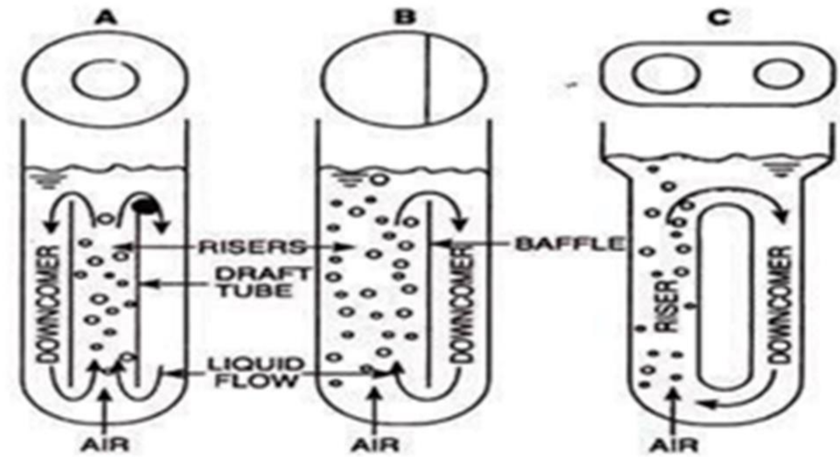


FIG. 39.2. Airlift fermentor. (A) Draft-tube internal loop configuration, (B) a split cylinder device, and (C) an external loop device.

AIRLIFT BIOREACTORS APPLICATION

- Airlift bioreactors are commonly employed for aerobic bioprocessing technology.
- They ensure a controlled liquid flow in a recycle system by pumping.
- Due to high efficiency, airlift bioreactors are sometimes preferred e.g., methanol production, waste water treatment, single-cell protein production.
- In general, the performance of the airlift bioreactors is dependent on the pumping (injection) of air and the liquid circulation.

CONTINUOUS STIRRED TANK BIOREACTORS

- A continuous stirred tank bioreactor consists of a cylindrical vessel with motor driven central shaft that supports one or more agitators (impellers).
- The shaft is fitted at the bottom of the bioreactor.
- The number of impellers is variable and depends on the size of the bioreactor i.e., height to diameter ratio, referred to as aspect ratio.
- The aspect ratio of a stirred tank bioreactor is usually between 3-5. However, for animal cell culture applications, the aspect ratio is less than 2.
- The diameter of the impeller is usually 1/3 rd of the vessel diameter.
- The distance between two impellers is approximately 1.2 impeller diameter. Different types of impellers (Rustom disc, concave bladed, marine propeller etc.) are in use.
- In stirred tank bioreactors or in short stirred tank reactors (STRs), the air is added to the culture medium under pressure through a device called sparger.
- The sparger may be a ring with many holes or a tube with a single orifice.
- The sparger along with impellers (agitators) enables better gas distribution system throughout the vessel.
- The bubbles generated by sparger are broken down to smaller ones by impellers and dispersed throughout the medium.
- This enables the creation of a uniform and homogeneous environment throughout the bioreactor.

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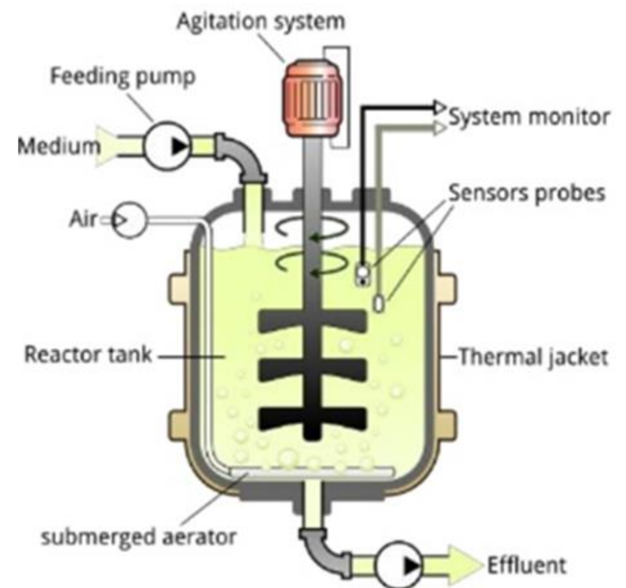
- This enables the creation of a uniform and homogeneous environment throughout the bioreactor.

Advantages of STRs:

There are many advantages of STRs.

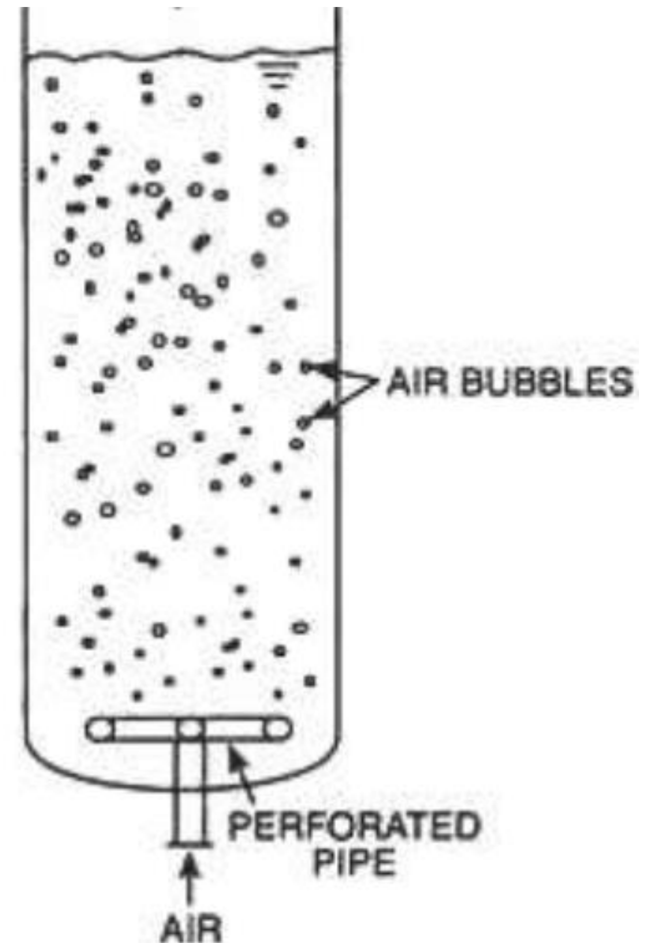
- The efficient gas transfer to growing cells.
- Good mixing of the contents and flexible operating conditions, besides the commercial availability of the bioreactors.



General structure of a continuous stirred-tank type bioreactor

Bubble Column Fermenter

- Bubble column fermenter is usually cylindrical with an aspect (height to diameter) ratio of 4-6.
- Gas is sparged at the base of the column through perforated pipes,perforated plates,or sintered glass or metal micro-porous spargers.
- O2 transfer,mixing and other performance factors are influenced mainly by the gas flow rate and the properties of the fluid.
- Internal devices such as horizontal perforated plates,vertical baffles and corrugated sheet packing,s may be placed in the vessel to improve mass transfer and modify the basic design.
- One exception is the axial mixing performance.
- For a given gas flow rate,the mixing improves with increasing vessel diameter.



- In the bubble column bioreactor, the air or gas is introduced at the base of the column through perforated pipes or plates, or metal micro porous spargers.
- The flow rate of the air/gas influences the performance factors —O2 transfer, mixing.
- The bubble column bioreactors may be fitted with perforated plates to improve performance.
- The vessel used for bubble column bioreactors is usually cylindrical with an aspect ratio of 4-6 (i.e., height to diameter ratio).

Fluidized Bed Bioreactors:

- Fluidized bed bioreactor is comparable to bubble column bioreactor except the top position is expanded to reduce the velocity of the fluid.
- The design of the fluidized bioreactors (expanded top and narrow reaction column) is such that the solids are retained in the reactor while the liquid flows out.
- These bioreactors are suitable for use to carry out reactions involving fluid suspended biocatalysts such as immobilized enzymes, immobilized cells, and microbial flocs.

- For an efficient operation of fluidized beds, gas is spared to create a suitable gas-liquid-solid fluid bed.
- It is also necessary to ensure that the suspended solid particles are not too light or too dense (too light ones may float whereas to dense ones may settle at the bottom), and they are in a good suspended state.
- Recycling of the liquid is important to maintain continuous contact between the reaction contents and biocatalysts.
- This enable good efficiency of bioprocessing.

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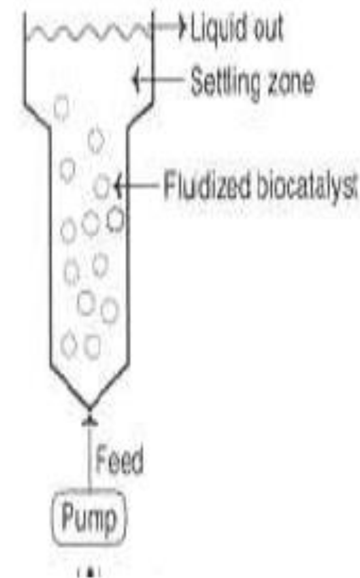
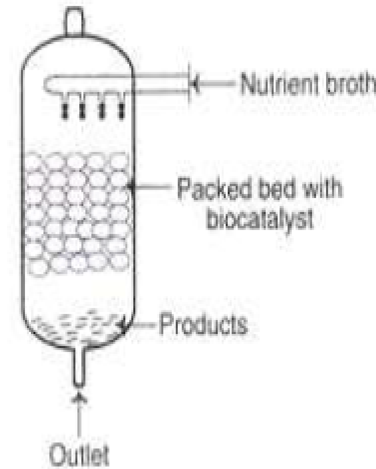


Packed Bed Bioreactors:

- A bed of solid particles, with biocatalysts on or within the matrix of solids, packed in a column constitutes a packed bed bioreactoı
- The solids used may be porous or non-porous gels, and they may be compressible or rigid in nature.
- A nutrient broth flows continuously over the immobilized biocatalyst.
- The products obtained in the packed bed bioreactor are released into the fluid and removed.
- While the flow of the fluid can be upward or downward, down flow under gravity is preferred.



- The concentration of the nutrients (and therefore the products formed) can be increased by increasing the flow rate of the nutrient broth.
- Because of poor mixing, it is rather difficult to control the pH of packed bed bioreactors by the addition of acid or alkali.
- However, these bioreactors are preferred for bioprocessing technology involving product-inhibited reactions.
- The packed bed bioreactors do not allow accumulation of the products to any significant extent.

PACKED BED BIOREACTORS

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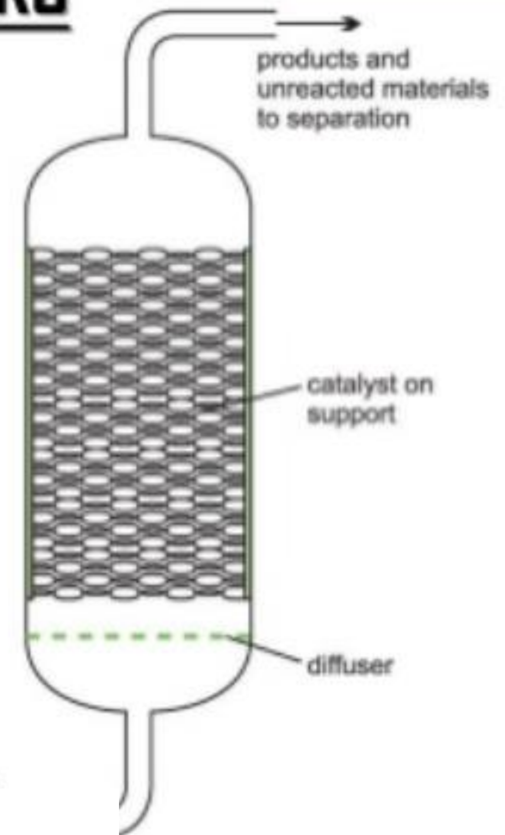
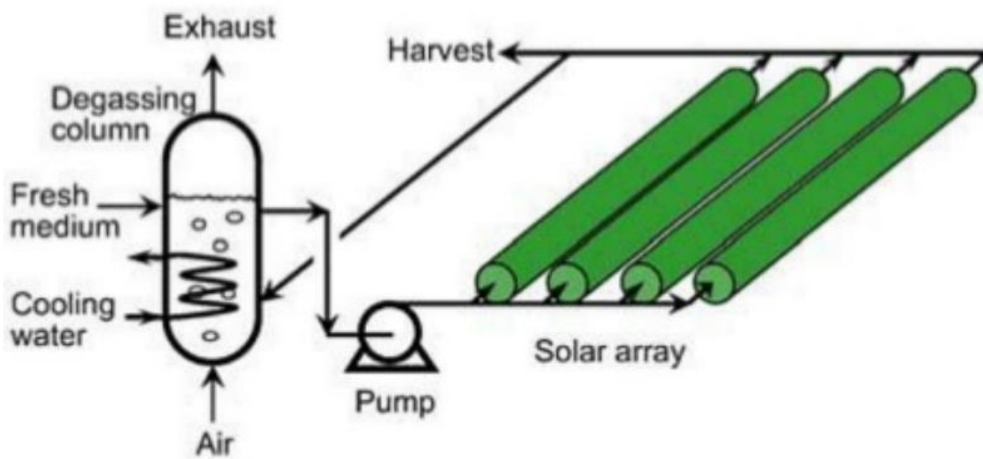


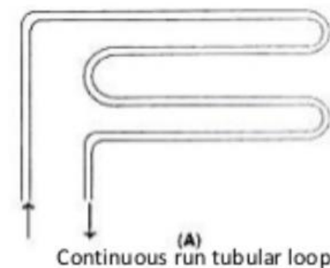
- A bed of solid particles, with biocatalysts on or within the matrix of solids, packed in a column constitutes a packed bed.
- The solids used may be porous or non-porous gels, and they may be compressible or rigid in nature.
- A nutrient broth flows continuously over the immobilised biocatalyst.
- The products obtained in the packed bed bioreactor are released into the fluid and removed.
- While the flow of the fluid can be upward or downward, down flow under gravity is preferred.
- The concentration of the nutrients (and therefore the products formed) can be increased by increasing the flow rate of the nutrient broth.
- Because of poor mixing, it is rather difficult to control the pH of packed bed bioreactors by the addition of acid or alkali.
- However, these bioreactors are preferred for bioprocessing technology involving product-inhibited reactions.
- The packed bed bioreactors do not allow accumulation of the products to any significant extent.

PHOTO-BIOREACTORS

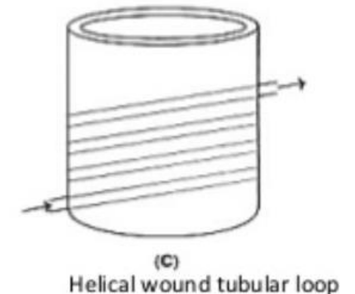
- These are the bioreactors specialised for fermentation that can be carried out either by exposing to sunlight or artificial illumination.
- Since artificial illumination is expensive, only the outdoor photo-bioreactors are preferred.
- Certain important compounds are produced by employing photo-bioreactors e.g., p-carotene, asthaxanthin.
- They are made up of glass or more commonly transparent plastic.
- The array of tubes or flat panels constitute light receiving systems (solar receivers).
- The culture can be circulated through the solar receivers by methods such as using centrifugal pumps or airlift pumps.
- It is essential that the cells are in continuous circulation without forming sediments.
- Further adequate penetration of sunlight should be maintained.
- The tubes should also be cooled to prevent rise in temperature.
- Photo-bioreactors are usually operated in a continuous mode at a temperature in the range of 25-40°C. Microalgae and cyanobacteria are normally used.
- The organisms grow during day light while the products are produced during night.



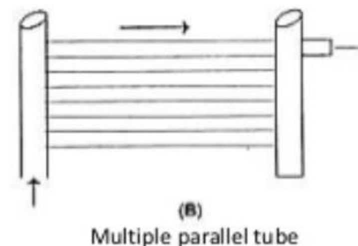
A tubular photobioreactors with parallel run horizontal tubes.



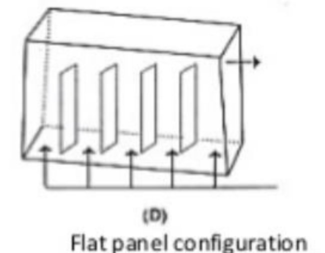
(A)
Continuous run tubular loop



(C)
Helical wound tubular loop



(B)
Multiple parallel tube



(D)
Flat panel configuration

Process scale up steps, laboratory scale, pilot plant and industrial scale

➢ **Definition** :-

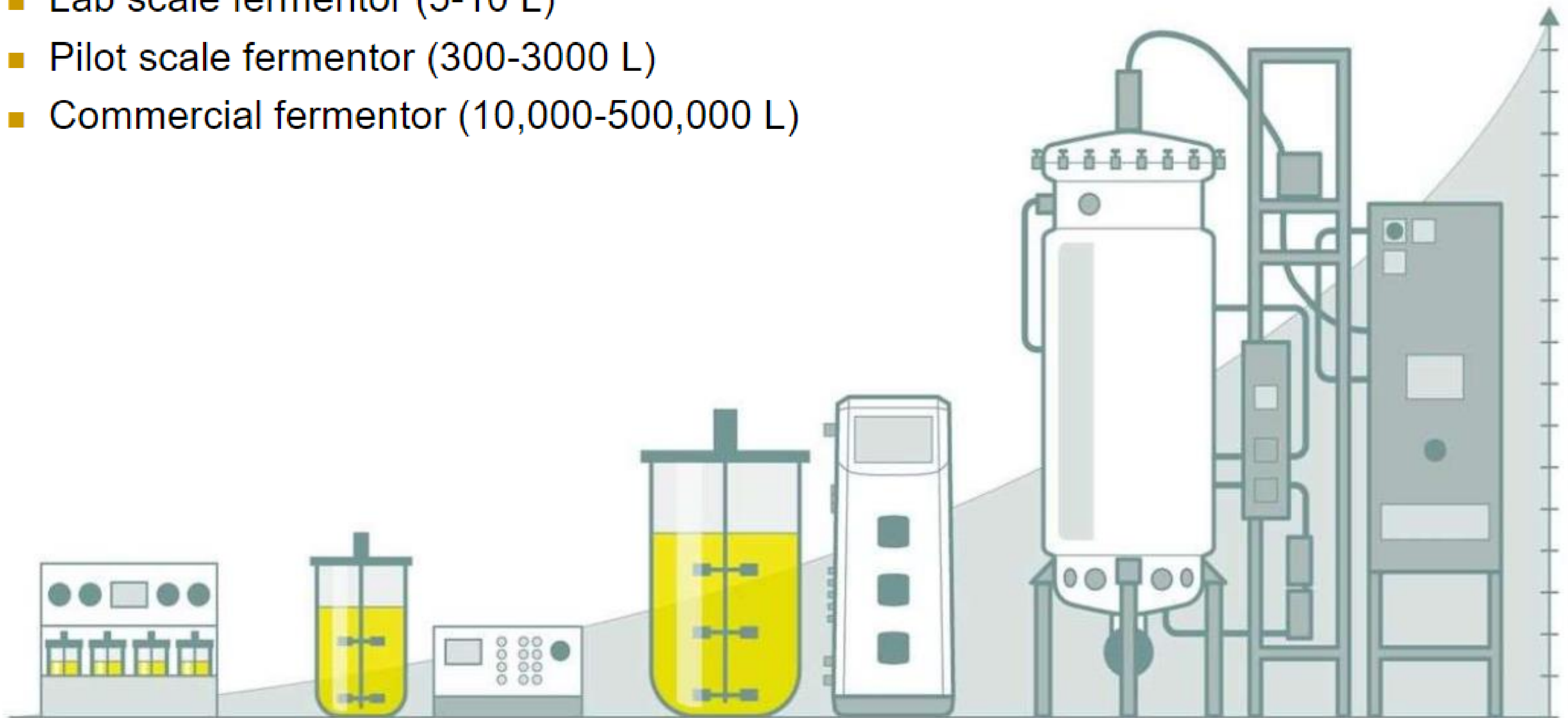
- Scale up means the art for designing of large scale apparatus or full size plant (prototype) using the data obtained from the laboratory studies

➢ **Objectives** **:-**

- To provide master manufacturing formula
- To identify the critical features of the process
- Evaluation, Validation and Finalization of process
- Guidelines for production and process control
- Review of the processing equipment
- To produce physically and chemically stable products

Stages needed for transferring an industrial process from the laboratory to the commercial fermentor

- Shake flash Experiments
- Lab scale fermentor (5-10 L)
- Pilot scale fermentor (300-3000 L)
- Commercial fermentor (10,000-500,000 L)



Laboratory scale

- To demonstrate process feasibility or generate design data for a process, then a mini plant may be more appropriate than a pilot plant.
- Includes all recycle streams and can be extrapolated easily
- Uses same components as the lab testing (pumps, etc.), which is often standardized and can be used in many other mini plants
- Operated continuously for weeks or months so some automation is required.
- Is used in combination with process modeling and simulation of the industrial scale process.

Pilot Scale Up

- **Scale up: The transfer of a process from small-scale laboratory equipment to large-scale commercial equipment**
- Pilot experiment
  - To test the feasibility of the lab scale fermentation process in a semi-industrial scale
  - Pilot fermentors normally have a size ranging from 100 L to 10,000 L, depending on the products to be mass produced later.

Pilot scale fermenter

- A **pilot scale fermenter** is a small industrial system, which is operated to generate information about the behavior of the system for use in design of larger facilities.
- Pilot plant is a relative term in the sense that plants are typically smaller than full-scale production plants, but are built in a range of sizes.

Pilot scale

- Pilot scale means an experimental system that represents the part it corresponds to in an industrial unit.
- A pilot plant allows investigation of a product and process on an intermediate scale before large amounts of money are committed to full-scale production
- It is not possible to design a large complex food processing plant from laboratory data alone with any degree of success

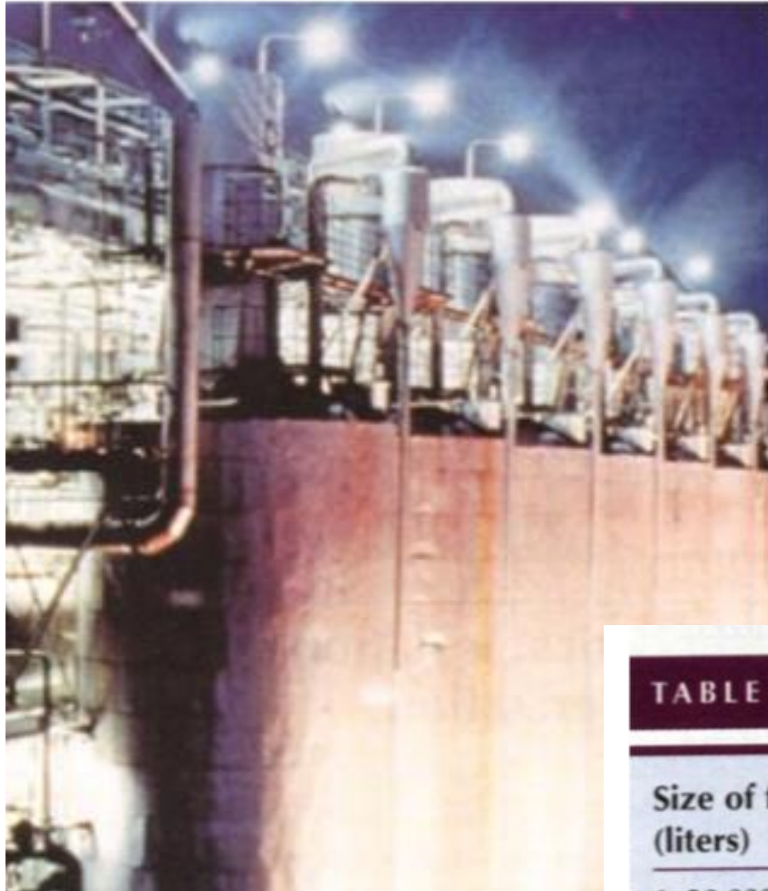
- Producing small quantities of product for sensory, chemical, microbiological evaluations, limited market testing or furnishing samples to potential customers, shelf-live and storage stability studies
- Determining possible salable by-products or waste stream requiring treatment before discharge
- Providing data that can be used in making a decision on whether or not to proceed to a full-scale production process and in the case of a positive decision, designing and constructing a full-size plant or modifying an existing plant

Problems emerging during the scale up

- As the size of the equipment is increased, the surface-volume ratio changes
- Large fermentor has much more volume for a given surface area, it is obviously more difficult to mix the big tank than the small flask
- In scale up studies on aerobic fermentations, oxygen rate in the fermentor is best kept constant as the size of the fermentor is increased.

  - How to keep DO constant?
    - Increase stirring rate
    - Increase air pressure
    - Use pure oxygen
    - Increase air inlet

Industrial Scale up



- To transfer the pilot scale results into a commercially feasible production setting.
- Fermentor sizes range from 100 L to 500,000 L, depending on products.

TABLE 12.2 Fermentor sizes for various industrial processes

Size of fermentor (liters)	Product
1–20,000	Diagnostic enzymes, substances for molecular biology
40–80,000	Some enzymes, antibiotics
100–150,000	Penicillin, aminoglycoside antibiotics, proteases, amylases, steroid transformations, amino acids
200,000–500,000	Amino acids (glutamic acid)

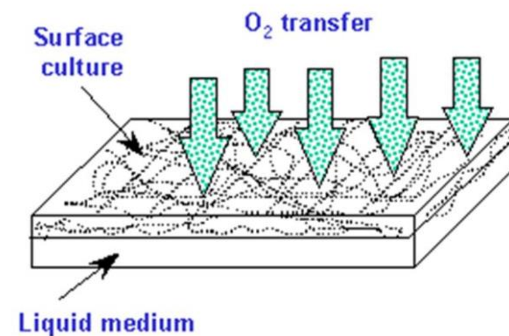
Types of Culture

- Based on phase of fermentation medium
  - Liquid Phase
    - Surface Liquid Culture
    - Submerged Culture
  - Solid Phase
- Based on mode of operation of fermentation process
  - Batch
  - Continious
    - Chemostat
    - Turbidostat
  - Fed -Batch

SURFACE CULTURE METHOD

Liquid surface culture process: -Generally preferred for fungus, especially mold based production process. Alluminium or stainless steel shallow pans (5-20 cms deep) or trays are used. The sterilized medium usually contains media and salts. The fermentation is carried out by blowing the fungal spores of production strain over the surface of the solution. Surface culture is used for production of citric acid and Penicillin. Spore germination occurs within 24 hours and a white mycelium grows over the surface of the solution within 4 days. This process is carried out to maximize the surface of substrate exposed to the air. The liquid can be drained off and any portion of mycelial mat left becomes submerged and inactivated. Required products are obtained by suitable downstream processing of the media.

***Aspergillus niger* mycelia**



Submerged fermentation:

- In the submerged process, the substrate used for fermentation is always in liquid state which contains the nutrients needed for growth.
- The fermentor which contains the substrate is operated continuously and the product biomass is continuously harvested from the fermenter by using different techniques then the product is filtered or centrifuged and then dried.
- Submerged fermentation is a method of manufacturing biomolecules in which enzymes and other reactive compounds are submerged in a liquid such as alcohol, oil or a nutrient broth.

Submerged fermentation / liquid fermentation

- Submerged fermentation is the techniques of cultivation of microorganism in liquid broth which breaks down the nutrient to release the desired bio-active compound into solution.
- In this method, selected microorganism are grown in closed vessels containing a broth rich in nutrients and high concentration of oxygen.
- In SmF substrate are utilized quite rapidly hence need to be constantly replaced or supplemented with nutrients
- Bacteria that requires high moisture content or high water activity are best suited for submerged fermentation.

The process is used for a variety of purposes, mostly in industrial manufacturing

Applications:

- Submerged Fermentation (SmF)/Liquid Fermentation (LF) SmF utilizes free flowing liquid substrates, such as molasses and broths. The bioactive compounds are secreted into the fermentation broth.
- The substrates are utilized quite rapidly; hence need to be constantly replaced/supplemented with nutrients.
- This fermentation technique is best suited for microorganisms such as bacteria that require high moisture.
- An additional advantage of this technique is that purification of products is easier.
- SmF is primarily used in the extraction of secondary metabolites that need to be used in liquid form

What is Solid State Fermentation??

- Solid-state (substrate) fermentation (SSF) has been defined as the fermentation process occurring in the absence or near-absence of free water.
- SSF employs natural raw materials as carbon source such as cassava, barley, wheat bran, rice bran, sugarcane bagasse, cassava bagasse, various oil cakes (e.g. coconut oil cake, palm kernel cake, soybean cake, ground nut oil cake, etc), fruit pulps (e.g. apple pomace), corn cobs, saw dust, seeds (e.g. tamarind, jack fruit), coffee husk and coffee pulp, tea waste, spent brewing

Advantages of solid-state fermentation

Down

- Low-cost media / Simple technology / Low capital costs / Reduced energy requirements / Low waste-water output
- Higher and reproducible product yields / Concentrated substrate / Smaller fermentation vessels
- Seed tanks are unnecessary, and spore inocula may be used
- Low moisture reduces the problem of contamination
- Conditions for fungal growth are similar to those in natural habitats
- Can be used to provide low-shear environments for shear-sensitive mycelial organisms
- Products may be incorporated directly into animal feeds

Solid state fermentation

- Solid state fermentation has been defined as "the fermentation process occurring in the absence or near absence of free water utilizing the solid substrate".
- It is a biomolecule manufacturing process used in the food, pharmaceutical, cosmetic, fuel and textile industries. These biomolecules are mostly metabolites generated by microorganisms grown on a solid support selected for this purpose.
- This technology for the culture of microorganisms is an alternative to liquid or submerged fermentation, used predominantly for industrial purposes

Disadvantages of solid-state fermentation

Down

- Slower microbial growth
- Problems with heat build-up
- Continuous agitation or rotation may involve high power requirements
- The addition of water in early fermentation stages may increase the risk of bacterial contamination
- Difficult scale-up and considerable developmental work
- Difficult control of the environment within the bioreactors, particularly the simultaneous maintenance of optimal temperature and moisture
- Agricultural substrates may require some kind of mechanical, chemical or biological pretreatment processing

ADVANTAGES OF SOLID STATE FERMENTATION OVER SUBMERGED FERMENTATION

- 1. HIGHER VOLUMETRIC PRODUCTIVITY.
- 2.USUALLY SIMPLER WITH LOWER ENERGY REQUIREMENTS
- 3. MIGHT BE EASIER TO MEET AERATION REQUIREMENTS
- 4. RESEMBLES THE NATURAL HABITAT OF SOME FUNGI AND BACTERIA
- 5. EASIER DOWNSTREAM PROCESSING.

APPLICATION OF SOLID STATE FERMENTATION

- **A. Production of Enzymes by Solid State fermentation-**

  protease, lipase, cellulase, pectinase
- **B. Production of Organic Acids under Solid State Fermentation-**

  citric acid, lactic acid.

C. Secondary Metabolites production Under SSF Condition- antibiotics, quinolines, growth factors, terpenoides

D. Production of Biocontrol Agents under Solid State Fermentation- eg. Biocontrol agent- microbial agent, fungal agent. *Liagenidium giganteum* a fungal agent used for control of Mosquitoes.

E. Production of Biofuel by Solid State Fermentation

eg. ethanol

Submerged fermentation	Solid state fermentation
1. Fermentation may be carried out as batch or continuous 2. Medium is added in large vessel 3. Surface area to volume height ratio is very less 4. 5-10% of inoculums is added 5. Inoculum is usually in liquid form 6. Product used are usually high as compared to input cost	1. Fermentation may be carried out as batch 2. Medium is added in flat vessel or trays 3. Surface area to volume height ratio is very high 4. Less inoculum is added 5. Inoculum is usually sprayed on surface of medium 6. Product yield is comparatively less
7. Lesser space is required 8. Less contamination 9. If a batch get contaminated there is a loss of entire batch 10. Entire fermentation media is utilized by microorganism for growth and product fermentation 11. Aeration and agitation of system is possible by use of sparger and impeller	7. More space is required 8. More contamination 9. If a tray gets contaminated then there is a loss of only tray but not the batch 10. There is wastage of fermentation media 11. Aeration is usually carried out by passing sterile air and no agitation
12. Power consumption is high 13. Controlling parameters like temperature, pH is easy 14. Foaming occurs 15. Automation and use of computer is easy 16. Less labor required 17. eg penicillin, streptomycin production etc.	12. Power consumption is less 13. Controlling parameters like temperature, pH is difficult 14. Foaming doesn't occurs 15. Automation and use of computer is difficult 16. More labor required 17. eg Veniger, amylase production etc.

SUBTRATES

Table.1

Submerged fermentation (SmF)	Solid state fermentation (SSF)
➢ Soluble sugar ➢ Molasses ➢ Liquid media ➢ Fruit and vegetable juices ➢ Sewage / waste water	➢ Wheat bran ➢ Rice and wheat straw ➢ Fruit and vegetable waste ➢ Paper pulp ➢ Bagasses ➢ Coconut coir ➢ Synthetic media

Batch Cultures

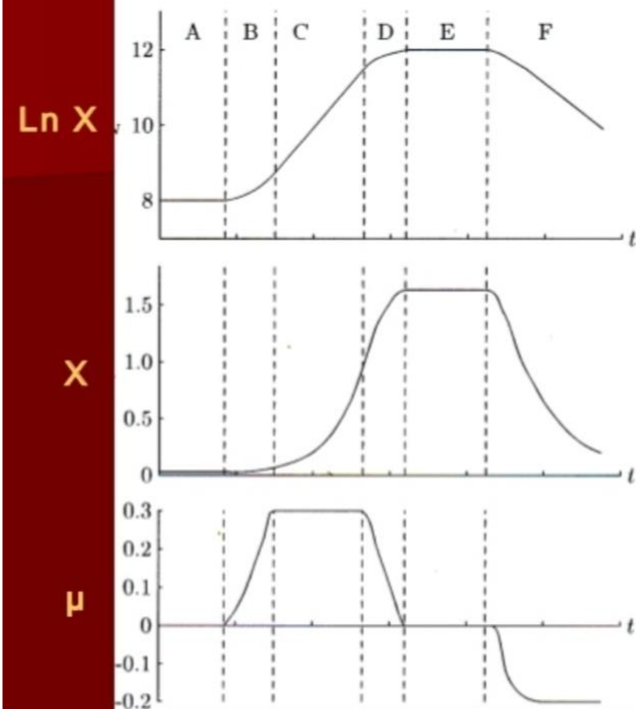
- In a batch operation, all necessary medium components and the inoculum are added at the beginning and not during period of fermentation.
- Therefore, their concentrations are not controlled but are allowed to vary as the living cells take them up.
- The products, be they intra- or extracellular, are harvested only at the end of the run.
- Basic controls for pH, temperature, dissolved oxygen, and foam are applied during the course of batch culture.
- The pH, dissolved oxygen, and temperature are normally held constant during the course of batch reactor operation.
- The only optimization parameters are the initial medium composition.
- However, profile optimizations of temperature and pH may lead to improved performance over the operations carried out at constant temperature and constant pH.

- Batch cultivation is closed system where there is no interaction between the system and the surrounding during the process. Except air during the aerobic cultivation.
- In Batch cultivation we prepare medium, sterilize it and inoculate the culture into the bioreactor.
- Allow the cells to grow and produce the product.
- Once the product formation reaches maximum harvest the fermentation broth.

How cells grow during Batch cultivation

After inoculating the medium and start measuring the biomass at different time intervals, you may find six different phases. They are

1. Lag phase
2. Accelerated growth phase
3. Exponential growth phase
4. Decelerated growth phase
5. Stationary phase
6. Death phase



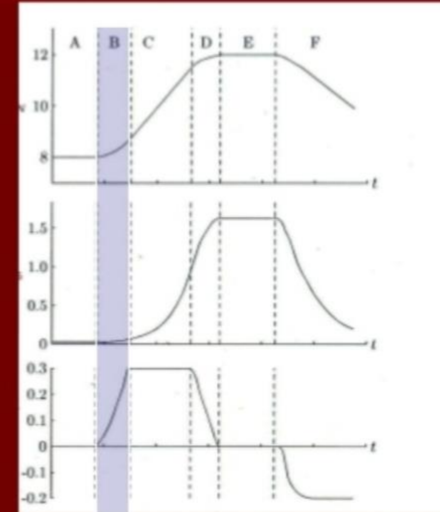
How to avoid lag phase

Lag phase is non productive period in the industrial fermentations. Hence minimizing it is essential.

- **The stage of culture from where the inoculum is drawn is important. Exponentially growing cells will have adequate concentrations of intermediates and intracellular pool of compounds. Hence if the inoculum is drawn from this stage they will not suffer dilution effect.**
- Size of the inoculum – If the size of the inoculum is large then the lag phase can be minimized. Generally 10% are used for yeast and mold and 5% for bacteria.
- Medium of inoculum should be same of that production medium.
- In certain cases such as recombinant *E.coli* cultivation to minimize plasmid loss higher percentage of inoculum will be used.

Accelerated growth phase

- At the end of Lag phase, when growth begins the division rate increase gradually and reaches a maximum value.
- The sp growth rate increases to maximum during this phase.



Exponential growth phase

- Cell division occurs in this phase.
- Often cell dry weight is used for cell concentration. During exponential phase we write as

$$dX/dt = \mu X$$

Where μ - Specific growth rate

X- cell dry weight

Rearranging and integrating the eqn

$$\int_{X_o}^{X} \frac{dX}{X} = \mu \int_0^t dt$$

$$\ln X\Big|_{X_o}^{X} = \mu t\Big|_0^t$$

$$\ln\left(\frac{X}{X_o}\right) = \mu t$$

$$X = X_o e^{\mu t}$$

Other phases of growth

- The end of the exponential phase occur when any of the essential nutrients is depleted or toxic metabolite accumulated in the system. During this phase the growth rate declines.
- Stationary phase will follow this phase. The length of stationary phase may vary with cell type, previous growth conditions etc., In certain cases the product formation will occur during this phase
- Following this is the death phase where the cells will start to lyse and the cell density decreases.

5.7. GROWTH YIELDS

When microbial growth is limited by low concentration of required nutrient, the final net growth or yield of cells increases with the initial amount of the limiting nutrient present. The rate of growth also increases with nutrient concentration but in a hyperbolic manner. The shape of the curve reflects the rate of nutrient uptake by microbial transport proteins.

When the nutrient quantity limits the production of bacteria, it is possible to define a growth yield constant (Y). The growth yield constant is the amount of dry weight of cells produced per weight of nutrient used

$$Y = \frac{\text{mass of microorganism formed}}{\text{mass of substrate consumed}}$$

The molar growth yield constant is Y_m, which is the dry weight of the cells produced (in grams) per mole of substrate used. For example, the $Y_{glucose}$ for aerobically growing cells is about 0.5, which means about 50% of the sugar is converted to cell material and 50% oxidised to CO_2. For certain sugars and bacteria, the efficiency of conversion of cell material can be much lower (e.g, 20%). In dilute media, some bacteria are able to increase their efficiency and assimilate upto 80% of the sugar acquired.

Continuous Cultures

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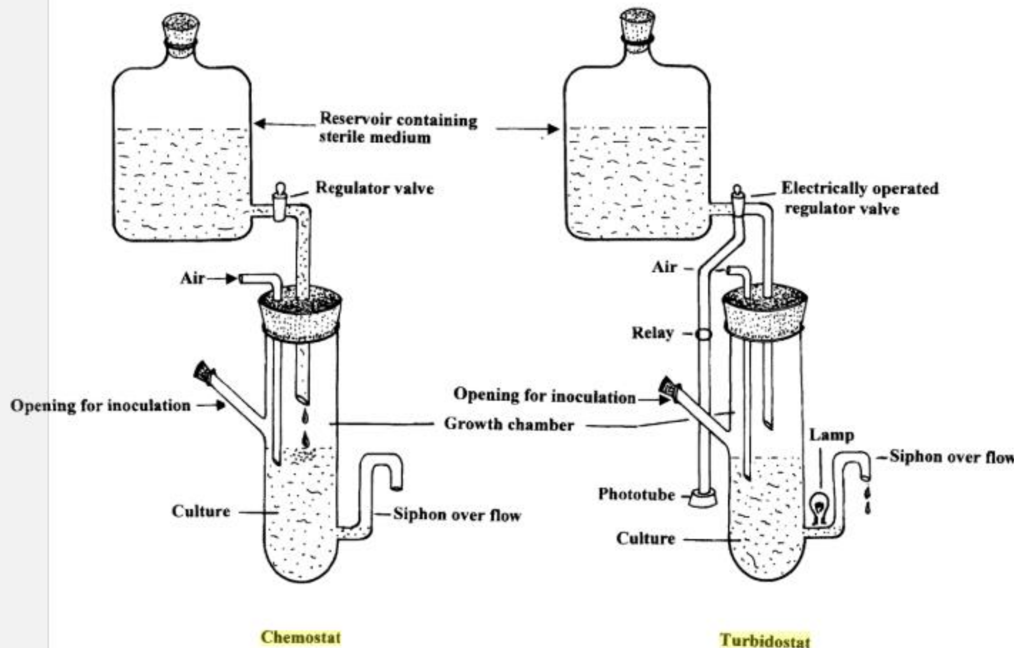
- In a continuous operation, one or more feed streams containing the necessary nutrients are fed continuously, while the effluent stream containing the cells, products, and residuals is continuously removed.
- A steady state is established by maintaining an equal volumetric flow rate for the feed and effluent streams.
- In so doing, the culture volume is kept constant, and all nutrient concentrations remain at constant steady state values.
- Continuous reactor operations are common in chemical industries. With the exception of single-cell protein production, certain beer production, and municipal waste treatment processes, continuous cultures have not been adopted widely by industry.
- It is not a dominant mode of industrial operation primarily because of the difficulty in maintaining sterility (contamination by other organisms) and protecting against phage attacks or mutations and because often, steady state operations are found to yield poorer results than dynamic operations, for reasons not yet fully understood.



CONTINUOUS GROWTH KINETICS

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- The actual growth rate depends not only on the volumetric flow rate of the medium into the reactor, but also on the dilution rate(D)

$$D = F/V$$

The net change in the cell concentration over a period of time may be expressed as:

$$dX/dt = \text{rate of growth in reactor} - \text{rate o loss from reactor}(\mu X - Dx)$$

Under steady state conditions, the rate of growth = rate of loss

$$dX/dt = 0$$

Therefore, $\mu X = DX$ & $\mu = D$

- For any given dilution rate, under steady state conditions, the residual substrate concentration in the reactor can be predicted by substituting D for μ in the Monod equation

$$\mathbf{D = \mu_{max}\, S_r / K_s + S_r}$$

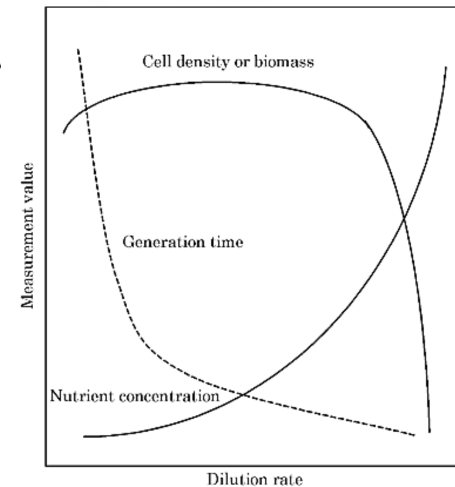
where S_r = steady state residual substrate concentration in the reactor at the fixed dilution rate. Rearrangement gives,

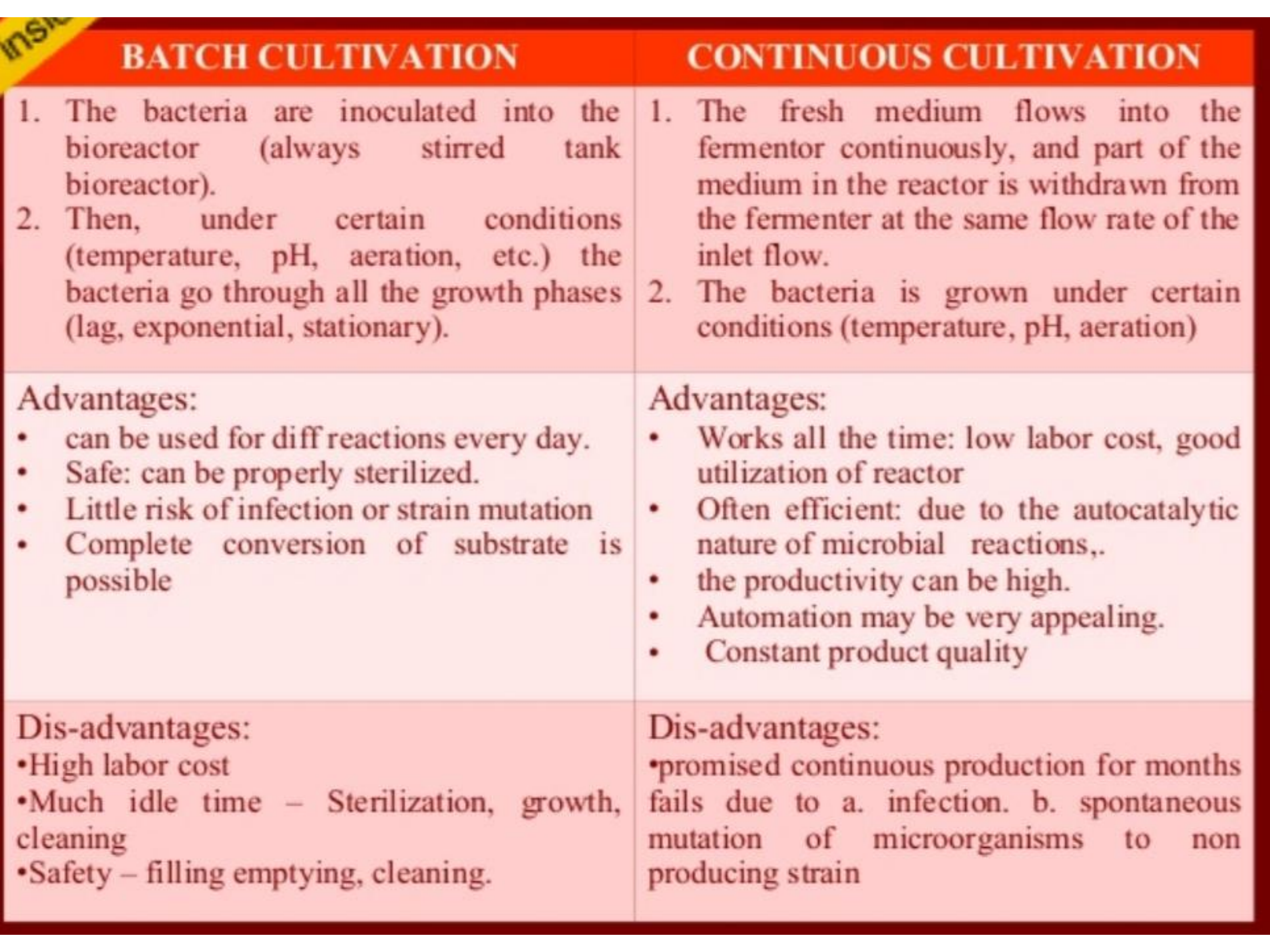
$$D(K_s + S_r) = \mu_{max}\, S_r \text{ or } DK_s + DS_r = \mu_{max}\, S_r$$

Dividing by S gives,

$$DK_s / S_r + D = \mu_{max}$$

hence, $\mathbf{S_r = DK_s / \mu_{max} - D}$



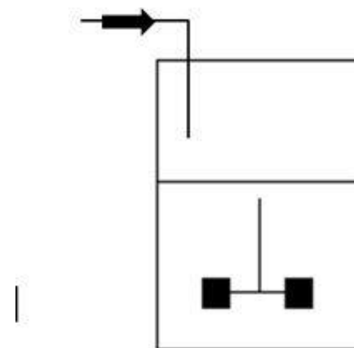


BATCH CULTIVATION	CONTINUOUS CULTIVATION
1. The bacteria are inoculated into the bioreactor (always stirred tank bioreactor). 2. Then, under certain conditions (temperature, pH, aeration, etc.) the bacteria go through all the growth phases (lag, exponential, stationary).	1. The fresh medium flows into the fermentor continuously, and part of the medium in the reactor is withdrawn from the fermenter at the same flow rate of the inlet flow. 2. The bacteria is grown under certain conditions (temperature, pH, aeration)
Advantages: • can be used for diff reactions every day. • Safe: can be properly sterilized. • Little risk of infection or strain mutation • Complete conversion of substrate is possible	Advantages: • Works all the time: low labor cost, good utilization of reactor • Often efficient: due to the autocatalytic nature of microbial reactions,. • the productivity can be high. • Automation may be very appealing. • Constant product quality
Dis-advantages: •High labor cost •Much idle time – Sterilization, growth, cleaning •Safety – filling emptying, cleaning.	Dis-advantages: •promised continuous production for months fails due to a. infection. b. spontaneous mutation of microorganisms to non producing strain

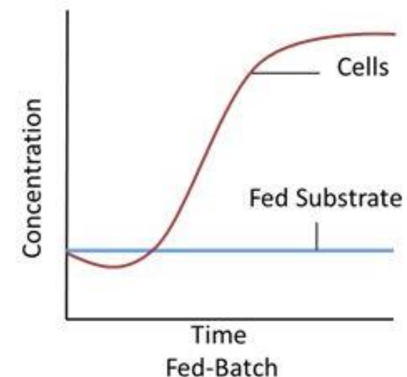
Fed-Batch Cultures

Clip slide

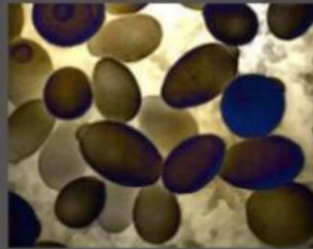
- A fed-batch culture is a semi-batch operation in which the nutrients necessary for cell growth and product formation are fed either intermittently or continuously via one or more feed streams during the course of an otherwise batch operation.
- The culture broth is harvested usually only at the end of the operational period, either fully or partially (the remainder serving as the inoculum for the next repeated run).
- This process may be repeated (repeated fed-batch) a number of times if the cells are fully viable and productive.
- Thus, there are one or more feed streams but no effluent during the course of operation.
- Sources of carbon, nitrogen, phosphates, nutrients, precursors, or inducers are fed either intermittently or continuously into the culture by manipulating the feed rates during the run. The products are harvested only at the end of the run.
- Therefore, the culture volume increases during the course of operation until the volume is full.
- Thereafter, a batch mode of operation is used to attain the final results. Thus, the fed-batch culture is a dynamic operation.
- By manipulating the feed rates, the concentrations of limiting nutrients in the culture can be manipulated either to remain at a constant level or to follow a predetermined optimal profile until the culture volume reaches the maximum, and then a batch mode is used to provide a final touch.
- In so doing, the concentration of the desired product or the yield of product at the end of the run is maximized. This type of operation was first called a *fed-batch culture* or *fed-batch fermentation*.



Batch

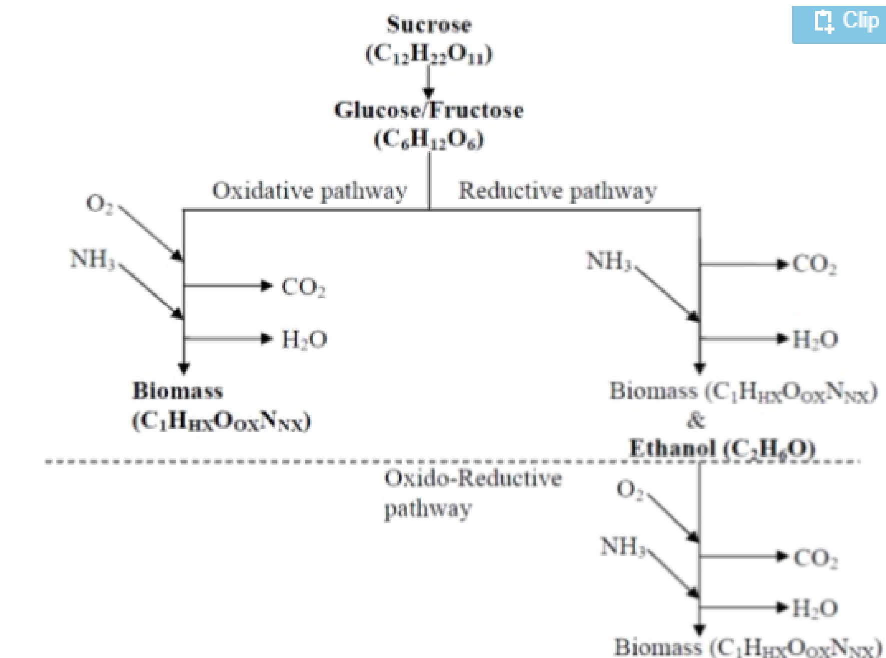


- *Marketed in the form of cake, powder or cream*
- *By-products are not required so --- directed towards max. biomass production*
- *Saccharomyces cerevisiae*
  - *Most commonly used organism*
  - *Unicellular*
  - *Rich in protein & vitamin B*
  - *Budding*
  - *Enzymes*
    - *Maltase; converts maltose to glucose*
    - *Invertase; sucrose to glucose & fructose*
    - *Zymase complex; sugars to CO_2 & ethanol*



Process Biochemistry

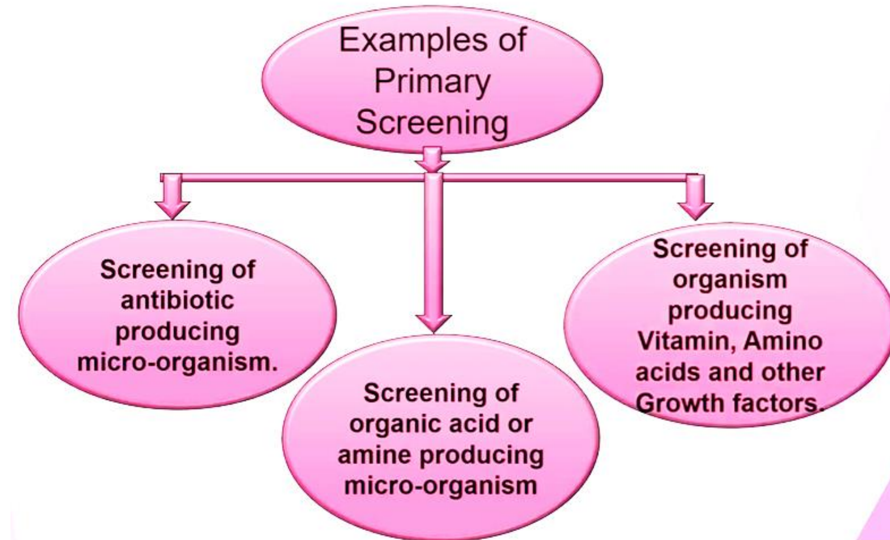
- Grow either in the absence or presence of O2
- Grows efficiently… O2 present
- Grows inefficiently… O2 not present
  - Produces ethanol in large quantity
- Fed-batch is best method
  - Incremntal feeding & high aeration



Screening allows the discarding of many valueless microorganisms, at the same time it allows the easy detection of the useful microorganisms that are present in the population in very less number

PRIMARY SCREENING TECHNIQUE

- Detection and isolation of industrially important micro-organism from mixed population using simple techniques is called as primary screening techniques.
- In primary screening technique simple methods are used to detect valuable micro-organism based on certain biochemical characters of microbes.
- Generally the sample is collected, serially diluted and isolated on suitable agar medium by specific technique.



2) PRIMARY SCREENING OF ANTIBIOTIC PRODUCING MICROORGANISMS

- Crowded plate technique is used for screening of antibiotic producing microorganisms.
- Does not give information about the sensitivity of antibiotics towards other microorganisms.
- Dilutions are made and then pouring and spreading of soil samples that give 300 to 400 or more colonies per plate.
- Colonies showing antibiotic activity are indicated by zone of inhibition around the colony .
- Such colonies are sub cultured and purified by streak before making stock cultures.
- The purified cultures are then tested to find the Microbial Inhibition Spectrum.

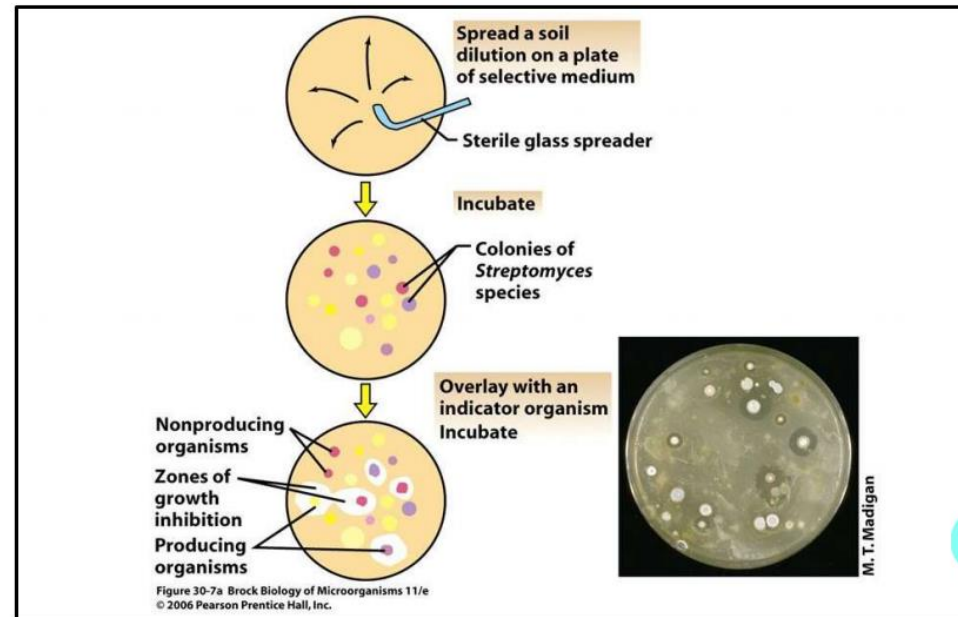


Figure 30-7a Brock Biology of Microorganisms 11/e
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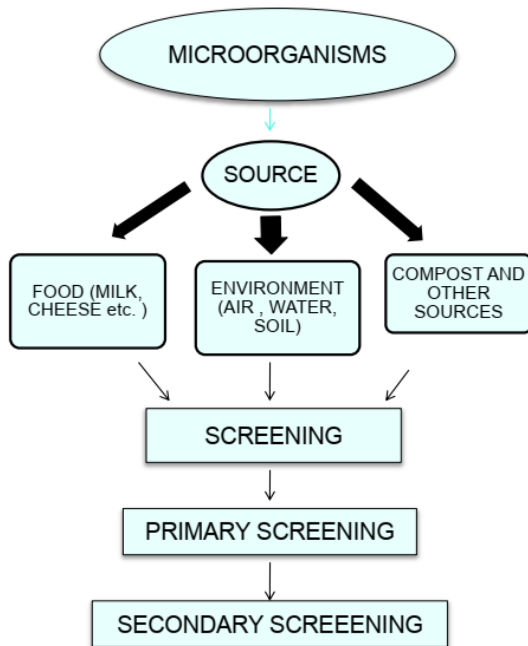
SCREENING

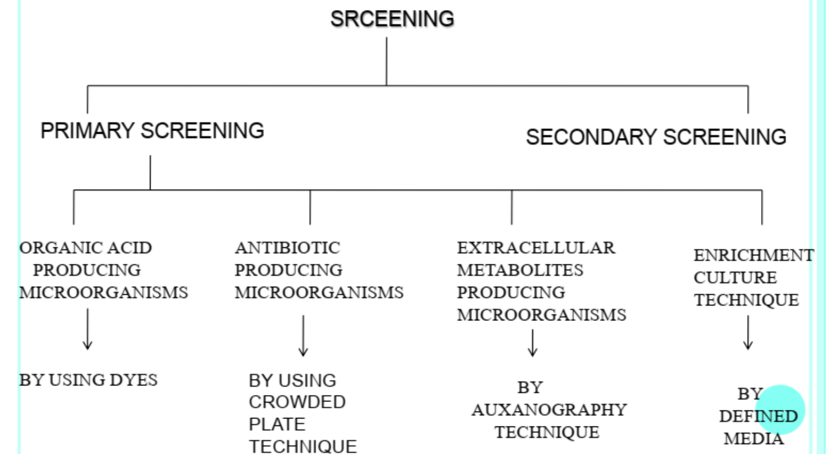
The procedure of isolation, detection , and separation of microorganisms of our interest from a mixed population by using highly selective procedures is called SCREENING



IMPORTANT THINGS TO BE CONSIDERED WHILE SCREENING :-

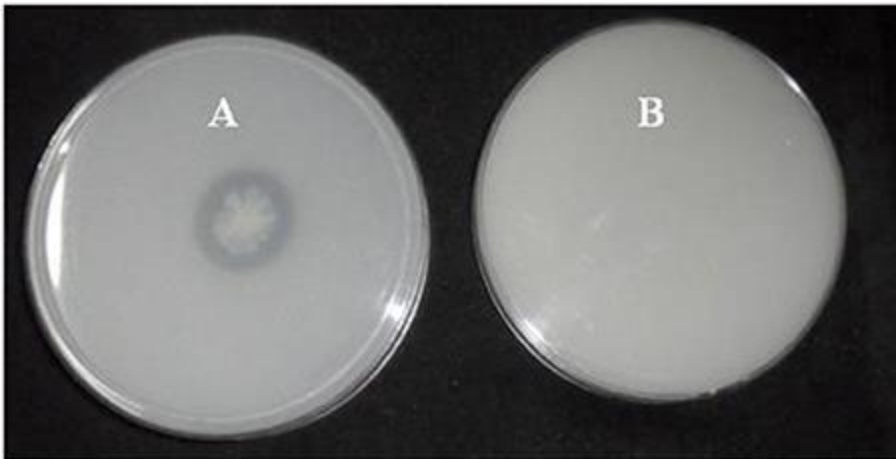
- 1.) CHOICE OF SOURCE - Samples from screening is taken from soil, water, air, milk, compost etc.
- 2.) CHOICE OF SUBSTRATE -Nutrients and growth factors should be supplied for growth of desired microorganism.
- 3.) CHOICE OF DETECTION - Proper isolation and detection of desired microorganisms is important

TYPES OF SCREENING



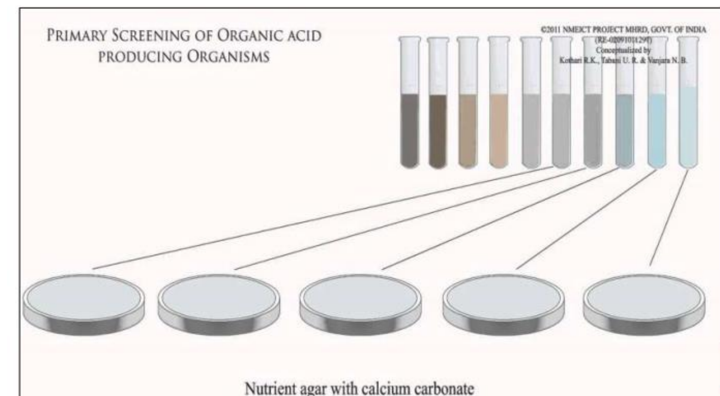
PRIMARY SCREENING

- It's a process for detection and isolation of microorganisms of our interest.
- Determines which microorganisms are able to produce a compounds.
- Does not provide much idea about the production or yield potential of microorganisms.
- It separate out only a few microorganisms, only few have commercial value while discards the valueless microorganisms .



1) PRIMARY SCREENING OF ORGANIC ACID PRODUCING MICROORGANISMS

- ❖The ph indicating **dyes** may be used for detecting microorganism that are capable of producing organic acids.
- ❖These dyes undergo color changes according to its ph.
- ❖Dyes such as **Neutral red, Bromothymol blue** are added to the poorly buffered nutrient agar media .
- ❖Colonies are subcultured to make stock culture.
- ❖Further testing is needed since inorganic acids, bases are also metabolic products of microbial growth.
- ❖ Incorporation of $CaCO_3$ in medium is also used to screen organic acid producing microbes on basis of formation of clear zone of dissolved $CaCO_3$ around the colony.



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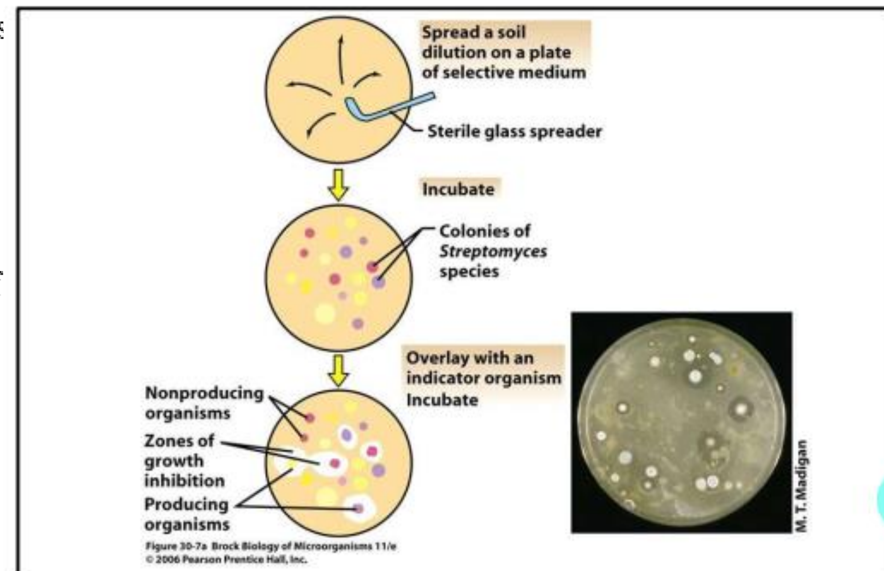


Auxanography technique

APM

- **Auxanography technique:** This technique is employed for the detection and isolation of microorganisms capable of producing certain extracellular substances such as growth stimulating factors like amino acids, vitamins etc.

This technique has two major steps are:-

A) Preparation of first plate:

- A filter paper is put across the bottom of petri dish.
- The nutrient agar is prepared and poured on the paper disc.
- Allowed to solidify
- Soil sample is diluted and proper dilutions are inoculated

Auxanography technique

B)Preparation of second plate:

- A minimal media lacking the growth factors is prepared and seeded with the test organism.
- The seeded medium is poured onto fresh petri plate and the plate allowed to set.
- The agar in first plate is then lifted and placed on the second plate without inverting.
- The growth factor produced on agar can diffuse into the lower layer containing test organism
- The zones of stimulated growth of test organism around colonies is an indication that organism produce growth factor extracellularly.

3) PRIMARY SCREENING EXTRACELLULAR METABOLITE PRODUCING MICROORGANISM

- ❖Auxanography technique is employed for detecting microorganisms able to produce growth factors , vitamins , amino acids etc. extracellularly.
- ❖ The 2 major steps are:-

A.) Preparation of first plate

- A filter paper strip is put across the bottom of petri dish.
- The nutrient agar is prepared and poured on the paper disc
- and allowed to solidify.
- Soil sample is diluted and proper dilutions are inoculated.

B.) Preparation of second plate

- A minimal media lacking the growth factors is prepared and seeded with the test organism.
- The seeded medium is poured onto fresh petri plate and the plate is allowed to set.

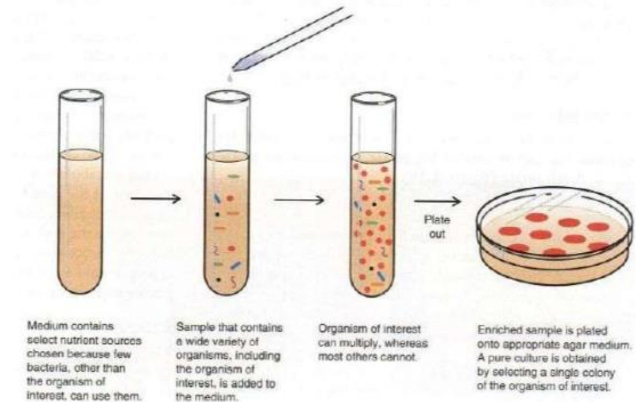
- ❖The agar in first plate is then lifted and placed on the second plate without inverting.
- ❖The growth factors produced on agar can diffuse into the lower layer containing test organism.
- ❖The zones of stimulated growth of test organism around colonies is an indication that organism produce growth factor extracellularly.

4) ENRICHMENT CULTURE TECHNIQUE

- ❖This was designed by Beijerinck to isolate the desired microorganism from heterogeneous microbial population.
- ❖ It consists of following steps :
  - a.) Nutrient broth is inoculated with microbial source material and incubated.
  - b.) A small portion of all inoculums is plated onto the solid medium and well isolated colonies are obtained.
  - c.) Suspected colonies from the plate are sub cultured on fresh media and subjected for further testing.

Enrichment cultures

Isolating an organism from natural sources



SECONDARY SCREENING

It's a systematic screening programme intended to isolate industrially important or useful microorganisms .

SOME IMPORTANT POINTS ASSOCIATED WITH SECONDARY SCREENING ARE:-

- It is useful in sorting of microorganisms that have real commercial value. The microorganisms having poor applicability in fermentation process are discarded.
- Provides the information whether the product formed by microorganisms is new or not. This may be accomplished by paper , thin layer, chromatographic technique.
- It should show whether the product possess physical properties such as UV light absorption or fluorescence or chemical properties that can be employed to detect the compound during use of paper chromatography.
- It is conducted on agar plates, in flasks or in small fermentor containing liquid media.
- It gives an idea about the economic position of the fermentation process involving the use of a newly discovered culture.
- It helps in providing information regarding the product yield potentials of different isolates.
- It determines the optimum conditions for growth or accumulation of a product associated with a particular culture.
- Chemical, physical and biological properties of a product are also determined during secondary screening. Moreover, it reveals whether a product produced in the culture broth occurs in more than one chemical form.
- It detects gross genetic instability in microbial cultures. This type of information is very important, since microorganisms tending to undergo mutation or alteration is some way may lose their capability for maximum accumulation of the fermentation products.
- It tells about the chemical stability of the fermentation product.
- It can be qualitative or quantitative in its approach.

DIFFERENCE BETWEEN PRIMARY SCREENING AND SECONDARY SCREENING

PRIMARY SCREENING	SECONDARY SCREENING
• Desired micro-organisms are isolated and detected. • Isolated micro-organisms are used for secondary screening. • Basic information about the isolated colony is obtained.	• Industrially Important organism are characterize by using highly selective procedures. • The organisms that are capable to give high yield of product by using cheap raw material are Screened. • Detail information of isolated colony is obtained and determined whether it can be used on industrial scale. • Here Capabilities and yield potential of microbes is determined • Here valueless organism are discarded and valuable organism that are capable to produce product on industrial scale are used further

Example of secondary screening –
ANTIBIOTIC PRODUCNING STREPTOMYCES SPECIES

1. Streptomyces isolates are streaked as a narrow band on nutrient agar plates are incubated .
2. Test organisms are then streaked from the edge of plates without touching streptomyceal isolate and then the plates are then incubated .
3. At the end of incubation, growth inhibitory zones for each organism are measured in millimeters .
4. Such organisms are again subjected for further testing by growing the culture in sterilized liquid media and incubated at constant temperature in a mechanical shaker.

Introduction

Clip slide

- To maintain pure culture for extended periods in a viable conditions, without any genetic change is referred as Preservation.
- During preservation most important factor is to stop microbial growth or at least lower the growth rate.
- Due to this toxic chemicals are not accumulated and hence viability of microorganism is not affected.

Objectives of Preservation

Clip slide

- To maintain isolated pure cultures for extended periods in a viable conditions.
- To avoid the contamination
- To restrict genetic change(Mutation)

Application of Preservation

- **Academic Use:**
- **Research Purpose:**
- **Fermentation Industry:**
- **Biotechnological Field:**

Once a microorganism has been isolated and grown in pure culture, it becomes necessary to maintain the viability and purity of the microorganism by keeping the pure culture free from contamination. Normally in laboratories, the pure cultures are transferred periodically onto or into a fresh medium (subculturing) to allow continuous growth and viability of microorganisms. The transfer is always subject to aseptic conditions to avoid contamination.

- Since repeated subculturing is time consuming, it becomes difficult to maintain a large number of pure cultures successfully for a long time. In addition, there is a risk of genetic changes as well as contamination. Therefore, it is now being replaced by some modern methods that do not need frequent subculturing. These methods include refrigeration, paraffin method, cryopreservation, and lyophilization (freeze drying).

- **5. Suspended Metabolism**
- **6. Storage at low temperature**
- **7. Freez dying**
- **8. Storage in silica gel**

- **Methods of Preservation**

- 1. Periodic transfer to fresh media (Subculturing)
- 2. Storage in sterile soil
- 3. Saline suspension
- 4.Oil overlay

Periodic transfer to fresh media

- Strains can be maintained by periodically preparing a fresh culture from the previous stock culture. The culture medium, the storage temperature, and the time interval at which the transfers are made vary with the species and must be ascertained beforehand. The temperature and the type of medium chosen should support a slow rather than a rapid rate of growth so that the time interval between transfers can be as long as possible.

- Many of the more common heterotrophs remain viable for several weeks or months on a medium like **Nutrient Agar**. The transfer method has the disadvantage of failing to prevent changes in the characteristics of a strain due to the development of variants and mutants.

Advantages

- 1. It is a simple method, any special apparatus are not required.
- Easy to recover the culture
- Disadvantage
- Risk of contamination is more
- It may be possible to change in genetic and biochemical characteristics

Lyophilization (Freeze-Drying)

Freeze-drying is a process where water and other solvents are removed from a frozen product **via sublimation.** Sublimation occurs when a frozen liquid goes directly to a gaseous state without entering a liquid phase. It is recommended using slow rates of cooling, as this will result in the formation of vertical ice crystal structures, thus allowing for more efficient water sublimation from the frozen product.

Freeze-dried products are hygroscopic and must be protected from moisture during storage. Under these conditions, the microbial cells are dehydrated and their metabolic activities are stopped; as a result, the microbes go into dormant state and retain viability for years. Lyophilized or freeze-dried pure cultures and then sealed and stored in the dark at 4°C in refrigerators.
Freeze-drying method is the most frequently used technique by culture collection centers. Many species of bacteria preserved by this method have remained viable and unchanged in their characteristics for more than 30 years.

Advantage of Lyophilization

Only minimal storage space is required; hundreds of lyophilized cultures can be stored in a small area.

Small vials can be sent conveniently through the mail to other microbiology laboratories when packaged in a special sealed mailing containers.

Lyophilized cultures can be revived by opening the vials, adding liquid medium, and transferring the rehydrated culture to a suitable growth medium.

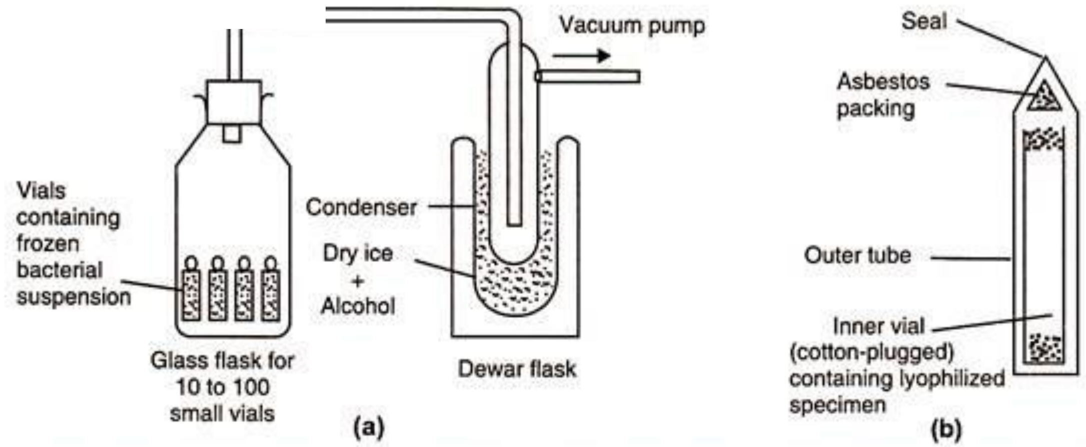


Fig. 2.16: (a) Small cotton-plugged vials containing frozen suspension of the microorganisms are placed in the glass-flask, which is attached to a condenser. The condensor is connected with a high-vacuum pump and this system brings about desiccaton of the cultures.

(b) After desiccation of the cultures as in (a) the vials are removed, placed individually in a large tube covered with asbestos packing and under vacuum.

Cryopreservation

- Cryopreservation (i.e., freezing in liquid nitrogen at -196°C or in the gas phase above the liquid nitrogen at -150°C) helps survival of pure cultures for long storage times.
- In this method, the microorganisms of culture are rapidly frozen in liquid nitrogen at -196°C in the presence of stabilizing agents such as glycerol or Dimethyl Sulfoxide (DMSO) that prevent the cell damage due to formation of ice crystals and promote cell survival.

- This liquid nitrogen method has been successful with many species that cannot be preserved by lyophilization and most species can remain viable under these conditions for 10 to 30 years without undergoing change in their characteristics, however this method is expensive.

Agar Slant Cultures:

- All microbiology laboratories preserve micro-organisms on agar slant. The slants are incubated for 24hr or more and are then stored in a refrigerator. These cultures are periodically transferred to fresh media. Time intervals at which the transfers are made which varies with the origin and condition of growth.

Agar Slant Culture Covered with Oil (Parafin Method):

- The agar slants are inoculated and incubated until good growth appears. They are then covered with sterile mineral oil to a depth of 1 cm above the tip of slant surface. Many bacterial species can preserved by covering the growth on the agar slants with steril mineral oil. Oil must cover the slants completely.Oil reduces the loss of water and also slow down the exchange of gas within organism and sorrounding
- This is a simple and most economical method of preserving bacteria and fungi where they remain viable for several years at room temperature. The layer of paraffin prevents dehydration of the medium and by ensuring an aerobic condition, the microorganism remain in dormant state.

Saline Suspension:

- Sodium chloride in high concentration is frequently an inhibitor of bacterial growth. Bacteria are suspended in 1% salt solution (sublethal concentration in screw cap tubes to prevent evaporation). The tubes are stored at room temperature. Whenever needed the transfer is made on agar slant.

Preservation by Drying in Vacuum:

- The organisms are dried over calcium chloride in vacuum and are stored in the refrigerator.

Media Used in Industrial Microbiology

- Use of appropriate growth medium is important to
  - Allow to harness the organism's full industrial potentials.
  - Prevent formation of toxic products.
- The basic nutrient requirements of Industrial media
  - All microbiological media must satisfy the needs of the organism in terms of
    - carbon, nitrogen, minerals, growth factors, and Water
  - In addition they must not contain materials which are inhibitory to growth.

Media Used in Industrial Microbiology

- **Media formulation**
  - Under laboratory conditions
    - Purified chemicals are used since volume is limited to a few litters
  - At industrial scale
    - Made with unpurified raw materials.

Criteria for the choice of raw materials used in industrial media

- In deciding the raw materials to be used in the production of a given products using designated microorganism the following factors should be taken into account.
  - Media cost must not be above the selling price.
  - The raw material must be readily available in order not to halt production.
  - Proximity of the user-industry to the site of production of the raw materials is important

- Ease of disposal of wastes resulting from the raw materials.
- The quality of the raw material in terms of its composition must be reasonably constant.
- Media must have Adequate chemical composition.
- The raw material must contain the precursors necessary for the synthesis of the finished product.

Fermentation media

- Most fermentations require
- 1-liquid media, often referred to as broth,
- 2-solid-substrate fermentations.
- In most industrial fermentation processes there are several stages where media are required.

1- several inoculum (starter culture) propagation steps,

2- pilot-scale fermentations

3-the main production fermentation.

- Where biomass or primary metabolites are the target product, the objective is to provide a production medium that allows optimal growth of the microorganism.
- For secondary metabolite production, such as antibiotics, their biosynthesis is not growth related. For this purpose, media are designed to provide an initial period of cell growth, followed by conditions optimized for secondary metabolite production. At this point the supply of one or more nutrients (carbon, phosphorus or nitrogen source) may be limited and rapid growth stopped

The main factors that affect the final choice of raw materials

- **1** Cost and availability
- **2** Ease of handling in solid or liquid forms, along with associated transport and storage costs, e.g. requirements for temperature control.
- **3** Sterilization requirements and any potential denaturation problems.
- **4** Formulation, mixing, complexing and viscosity characteristics that may influence agitation, aeration and foaming during fermentation and downstream processing stages.
- **5** The concentration of target product , its rate of formation and yield per gram of substrate utilized.
- **6** The levels and range of impurities, and the potential for generating further undesired products during the process.
- **7** Overall health and safety implications.

Carbon sources

- A carbon source is required for all biosynthesis leading to reproduction, product formation and cell maintenance.
- In most fermentations it also serves as the energy source.
- Carbon requirements may be determined from the biomass yield coefficient (*Y*), an index of the efficiency of conversion of a substrate into cellular material.

- $Y_{carbon(g/g)}$ = Biomass produced(g) / Carbon substrate utilized (g)
- For commercial fermentations the determination of yield coefficients for all other nutrients is usually essential.
- Various organisms may exhibit different yield coefficients for the same substrate due to the pathway by which the compound is metabolized.
- For example, *Saccharomyces cerevisiae* grown on glucose
- has biomass yield coefficients of 0.56 under aerobic condition and 0.12 g/g under anaerobic conditions

- Carbohydrates are traditional carbon and energy sources for microbial fermentations, although other sources may be used, such as alcohols, alkanes and organic acids. Animal fats and plant oils may also be incorporated into some media, often as supplements to the main carbon source.

Fermentation Media

- Major components
  - Carbon Source
  - Nitrogen source
- Minor components
  - Inorganic salts, vitamins, growth factors, anti-foaming agents, buffers, dissolved oxygen, other dissolved gases, growth inhibitors and enzymes

- Requirement of Nutrients depend upon type of organism used and type of fermentation that is carried out.
- Types of nutrients present in the fermentation media determine the yield of the product.
- There are two uses of fermentation media

1. Growth Media – It contains less nutrients and used for growth of micro-organism and preparation of Innoculum for fermentation.
2. Fermentation media – It contains large amount of nutrients this media is used in fermentation industries for production economically important products.

Role of fermentation media

- During fermentation process organisms enjoy luxurious metabolism with high amount of nutrients, optimum conditions.
- Due to presence of large amount of nutrients the micro-organism become hyperactive and consume excess nutrients and results in partial degradation of fermentation media.
- The waste product execrated by microbes may be the desired product.
- The substrate given to microbes should not reach inhibitory concentration levels as it may result death of cells.
- Water present in cell cytoplasm is also a important factor.
- Excess substrate may increase osmotic pressure and effect enzyme activities in a cell.
- Microbes excrete this excess substrate in the form of partially digested fermentation media.

Types of Fermentation Media

- Synthetic Media
- Crude media

Synthetic media

- The exact composition of nutrients is predetermined.
- Here level and concentration of nutrients can be controlled. Media can be re-designed
- Used in experiment related to effect of nutrients on growth of microbes and yield of product.
- Metabolic pathways and main ingredient that is involved in product formation can be determined.
- Synthetic media can be optimized as per our requirement.
- Product recovery is easy as all pure ingredients are used.
- It is expensive media and cannot be used on industrial scale as the process is not economical and profitable.

Crude Media

1)Crude media contains a rough composition of media.
2) It gives high yield of product and contains undefined sources of ingredients.
3) It contains high level of nutrients, vitamins, proteins, growth factors, anti-foaming agents and precursors.
4) It is important to ensure that crude media should not contain toxic substance.

Ingredients of Crude media

- Inorganic nutrients
- Carbon source
- Nitrogen source
- Growth factors
- Precursors
- Buffers

1) Inorganic nutrients – It contains salts containing anion and cations along with carbon source. Specific ions like magnesium ions, phosphates and sulphate are added to balance crude media.

2) Carbon source - Simple to complex carbohydrates are used. Different sugars, organic acids, fatty acids, proteins, peptides are added as a carbon source. In fermentation media crude source of carbon is added.
Examples -Black strap molasses, Corn molasses, Beet molasses, Starch, Corn, Rice, Rye, Milo, wheat, straws, wood waste, saw meal etc.

3) Nitrogen source - Non-proteolytic micro-organism need pure form of urea, ammonia and nitrate as a source of nitrogen. When fermentation organism is proteolytic in nature animal and plant raw material is used as a nitrogen source.
Examples - Distillery dried solubles, Casein, Cereal grains, peptones, yeast extract, hydrolysate, and soybean meal etc

4) Growth factor - Crude media constituents provides enough amount of growth factors so no extra addition of growth factor is required. If there is a lack of any kind if vitamins or nutrients, growth factors can be added to media. Examples are yeast extract, and beef extract.

5) Precursors – It is a crude constitute, Precursor in cooperate in the molecules of product without changing the final product. It improves yield and quality of the product. Example, Cobalt chloride is added fermentation of vitamin B12.

6) Buffers -Buffers are used to control drastic changes of pH. Sometimes, media components may act as buffers. For example, protein, peptides, amino-acids act as good buffers at neutral pH. Sometimes inorganic buffers like K_2HPO_4, KH_2PO_4, and $CaCO_3$ can be added as required. Generally, during the fermentation process, pH changes to acidic or alkaline pH. The cheapest and easily available buffer is $CaCO_3$.

Media Used in Industrial Microbiology

- Some raw materials used in compounding industrial media

a) Corn steep liquor

b) Pharmamedia

c) Distillers soluble

d) Soya bean meal

e) Molasses

f) Sulfite liquor

g) Other Substrates (alcohol, acetic acid, methanol, methane, and fractions of crude petroleum)

Corn Steep Liquor Corn steep liquor is a by-product of starch extraction from maize and its first use in fermentations was for penicillin production in the 1940s. The extract composition of the liquor varies depending on the quality of the maize and the processing conditions. Concentrated extracts generally contain about 4% (w/v) nitrogen, including a wide range of amino acids, along with vitamins and minerals.

Molasses

The byproducts of sugar industry include the beet and cane molasses. They are the concentrated syrups or mother liquors recovered at any of several steps in the sugar-refining process, and they are named depending on the particular step from which it is recovered. Blackstrap molasses prepared from sugarcane is the cheapest and most used sugar source for industrial fermentations. Blackstrap molasses is the mother liquor left after the crystallization of sugar and it contains approximately 52 per cent total sugars of sucrose in which about 30 per cent approximately is sucrose. When molasses is used as a fermentation component it should contain at least 50 per cent fermentable sugars.

The high-test or invert molasses is produced by partially inverting the whole cane juice to prevent sugar crystallization of sugars.

Sugar is partially hydrolysed to monosaccharide with heat and acid then neutralized and further concentrated without removal of any sugar. It contains approximately about 75-80 per cent sugar which is mostly the original sugar of the cane juice and some of which is partially hydrolyzed to D-glucose and D-fructose. It is preferable to blackstrap molasses because of the lower levels of non-fermentable solids including salts and un-fermentable sugars.

In addition to the sucrose, blackstrap molasses contains small amounts of complex polysaccharides invert sugars (due to the action of enzyme invertase), calcium organic acids (malic, citric, lactic, humic and acetic and propionic) nitrogen containing compounds like (aspartic and succinic acid) and alkali stable vitamins (niacin, riboflavin). The dark coloured nitrogen containing polymeric substances result from "browning" a reaction of the sugar with amino acids, heat and alkali are used in this processing. The overall composition of the various molasses differs according to the specific geographic area of production.

Pharmamedia

- Yellow fine powder made from cotton-seed embryo.
- It is used in the manufacture of tetracycline and some semi-synthetic penicillins.
- rich in protein, (56% w/v) and contains 24% carbohydrate, 5% oil, and 4% ash
- rich in calcium, iron, chloride, phosphorous, and sulfate.

Sulfite Liquor

- Sulfite liquor (also called waste sulfite liquor, sulfite waste liquor or spent sulfite liquor) is the aqueous effluent resulting from the sulfite process for manufacturing cellulose or pulp from wood.
- During the sulfite process, hemicelluloses hydrolyze and dissolve to yield the hexose sugars, glucose, mannose, galactose, fructose and the pentose sugars, xylose, and arabinsoe.
- Used as a medium for the growth of microorganisms after being suitably neutralized with $CaCO_3$ and enriched with ammonium salts or urea, and other nutrients.

Antifoam agents

High degree of aeration and agitation will result in foam formation.

Foaming reduces oxygen transfer. Air bubbles entrapped in the foam and again and again they recirculate in the medium. This will result in oxygen depleted bubbles residing in the system

To control foam antifoam agents are added.

- Most of the antifoams are surface tension lowering substances
- This will result in rigid bubble formation and resistance to oxygen transfer.
- Also antifoams in the liquid may favour coalescence of bubbles in freely moving areas which again will decrease oxygen transfer.
- OTR can be reduced dramatically even by factor of 10.

Disadvantages of Foaming:

1. Reduce process productivity since bursting bubbles can damage proteins,
2. Can result in loss of sterility if the foam escapes the bioreactor.
3. Leads to over-pressure if a foam-out blocks an exit filter.

Antifoam agent : soya bean oil , corn oil, lard oil and silicones (sterilized before adding)

AIR AND MEDIA STERILIZATION

PRINCIPLE

- Sterilization removes infecting micro-organisms it can also remove pathogenic micro-organisms or spoiling agents.
- Sterilization is accomplished either by chemical or physical means.
- Moist heat is a most common physical agent.
- It allows for satisfactory industrial sterilization.
- The other method of sterilization is the removal of infecting micro-organisms.
- This is done by filtration. Numerous type of filter papers are available for this purpose.
- It depends on the-
  (i)- The size of micro-organisms and
  (ii)-The retention efficiency of the filter.
- Usually sterilization of gases and biostatic fluids is done by filtration.

AIR AND MEDIA STERILIZATION

STERILIZATION OF MEDIA

- Usually media are sterilized before they are inoculated.
- Sterilization of media is decided by the chemical composition.
- Sterilization of media may be done by one of the following three methods-
  (i)-by boiling
  (ii)-by passing live steam
  (iii)-by subjecting the medium to steam under pressure(i.e. autoclaving)
- The classical technique of making the medium sterile by the use of steam may be carried out in two ways-

  (i)-batch wise in fermentor and
  (ii)-continuous sterilization

BATCH WISE IN FERMENTOR

- This is the simplest method of sterilizing production media.

- The vessel is equipped with a coil or jacket for heating and cooling.
- Also the agitator may be fitted to aid heat-exchange.
- It is needed to raise the temperature of the medium to 120^0C with steam to maintain this for a period of 20 minutes before cooling the system.
- There is an interconnecting pipeline between the batch the batch cooker and the fermentor for transferring the sterile medium from the cooker to steam sterilized fermentor.

ADVANTAGE

- The batch cooker method saves the production time, since the fermentor is unoccupied between two fermentor runs.

LIMITATION

- It occupies increased plant space.
- It involve higher cost of the additional equipment required, and
- It involves increased steam usages.

CONTINUOUS STERILIZATION

- This methods involves passing of production medium through a heat exchanger, a holding coil and a cooler.
- The temperature of medium undergoing sterilization is raised to the desired level in the heat exchanger.
- The medium is then passes on to a holding coil.

➢Where it is maintained at the sterilizing temperature for a predetermined time period.

➢Finally the medium is rapidly cooled by counter circulating it in the exchanger against the cool input medium, and then against cold water.

➢In continuous sterilization the temperature is higher than 120^{0}C.

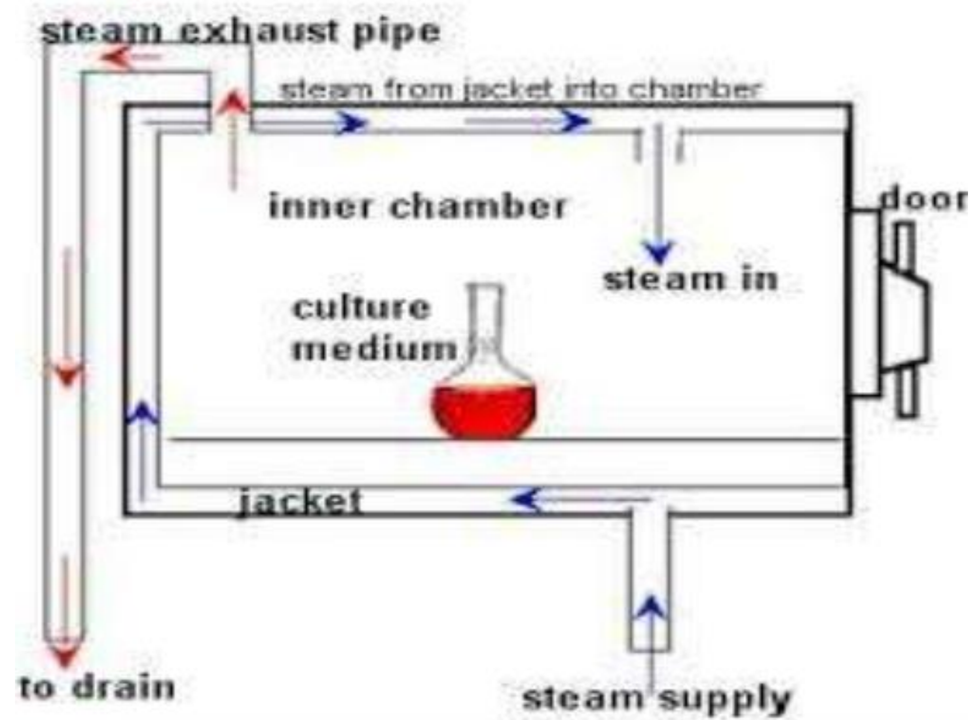
ADVANTAGES

➢It saves both production time and plant space.

➢It gives improved quality of the medium.

➢It involves some economy in steam cost.

➢It allows the use of lower sterilizing temperature or shorter holding period.



➢With aerobic fermentation continuous supply of sterile air is vital for successful fermentation.

➢Air can be sterilized by many methods namely-

(i)-filtration

(ii)-heat

(iii)-electrostatic repulsion

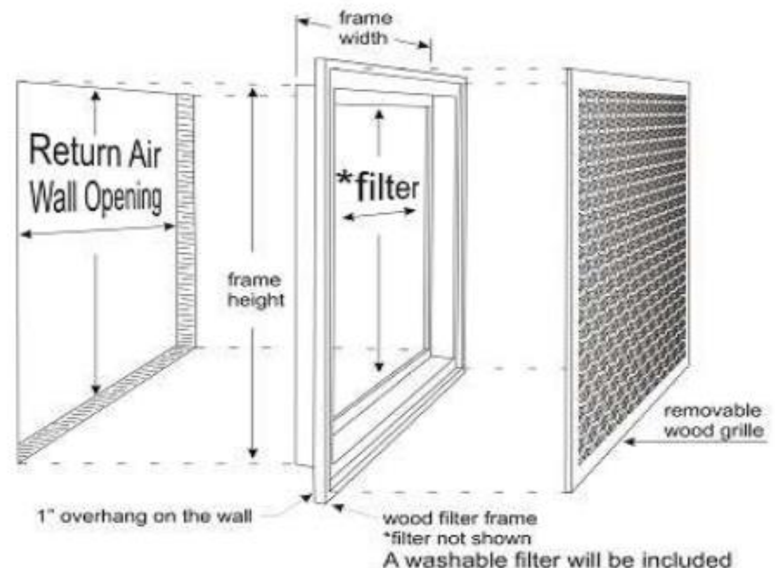
(iv)-U.V. light

(iv)-chemical agents

A I R S T E R I L I Z A T I O N

- The sterilization of air in fermentation industries is widely carried out by the filtration method.
- For sterilizing large volumes of air was studied by Terjesen and cherry.
- They used a performed slab wool 3 inches thick.
- The air velocity through the slab was kept below 1ft./sec. to avoid channeling through the slag wool material.

A I R S T E R I L I Z A T I O N



Strain Improvement/Development

❖ **Strain Improvement-** The Science and Technology of manipulating and improving microbial strains in order to enhance their metabolic capacities is known as Strain Improvement

Targets for industrial strain improvement

- **Increase product concentration:** (1) raise the gene dose; (2) break down the gene regulation (e.g. catabolite derepression, metabolite resistance); or (3) alter permeability to improve product export
- **Process improvement:** (1) decrease fermentation time; (2) be able to metabolize inexpensive substrates; (3) do not produce undesirable by products e.g. pigments or substance chemically related to the main product; (4) reduce oxygen needs; (5) decrease foaming; (6) tolerant to high concentrations of carbon or nitrogen sources; (7) resistant to phage …
- **New product:** Changes in the genotype of microorganisms can lead to the biosynthesis of new metabolites.

Immobilization of cells

- Immobilization of cells means the encapsulation of cells in culture by polymers like, sodium alginate, calcium alginate, collagen, poly styrene, agar or cellulose derivatives, so that cells are not able to divide but remain viable for so many weeks.
- One of the major problems in cell culture based process for secondary metabolite production is high production cost due to slow growth of cells, low product yield, genetic instability of the selected cells and intra cellular accumulation of the product. Some of these problems can be reduced by immobilized cell culture.
- In this technique cells are confined within a reactor system, preventing their entry into the mobile phase which carries the substrate and products/nutrients.

- Immobilization is only relevant where the production involves two stages; in the first stage conditions are optimized for biomass production by suspension culture and in the second stage conditions are optimized for product formation by immobilized cells.

The advantages of Immobilized cell culture are-

- It may enable prolonged use of biomass;
- By immobilization of cells the cell density in a bioreactor can be increased 2-4 times that in suspension cultures (10-30 g/l) and this enables the use of small reactors, reducing the cost of medium, equipment installation and downstream processing;
- The entrapped cells are protected against shear forces and, consequently, a simple bioreactor design may be used;
- It separates the cells from the medium and, therefore, if the product is extracellular it can simplify downstream processing;
- It uncouples growth and product formation which allows product optimization without affecting growth;

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