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PG and Department of Biotechnology

IInd M.Sc. Biotechnology – Semester - III

E-Notes (Study Material)

Core Course 9-: BIOPROCESS TECHNOLOGY
UNIT-II **Types of bioreactors: Submerged reactors, surface reactors, mechanically agitated reactors, non-mechanically agitated reactors. Design of fermenters. Production of citric acid, penicillin and insulin. Isolation and improvement of Industrially important Micro-organisms, Media for Industrial fermentation and Sterilization.**
Learning Objectives: Outline the basis of bioreactors
Course Outcome: Students will gain a comprehensive understanding of the basic principles underlying bioreactors processes, including the types of f bioreactors and their applications in various industries.

DEFINITION

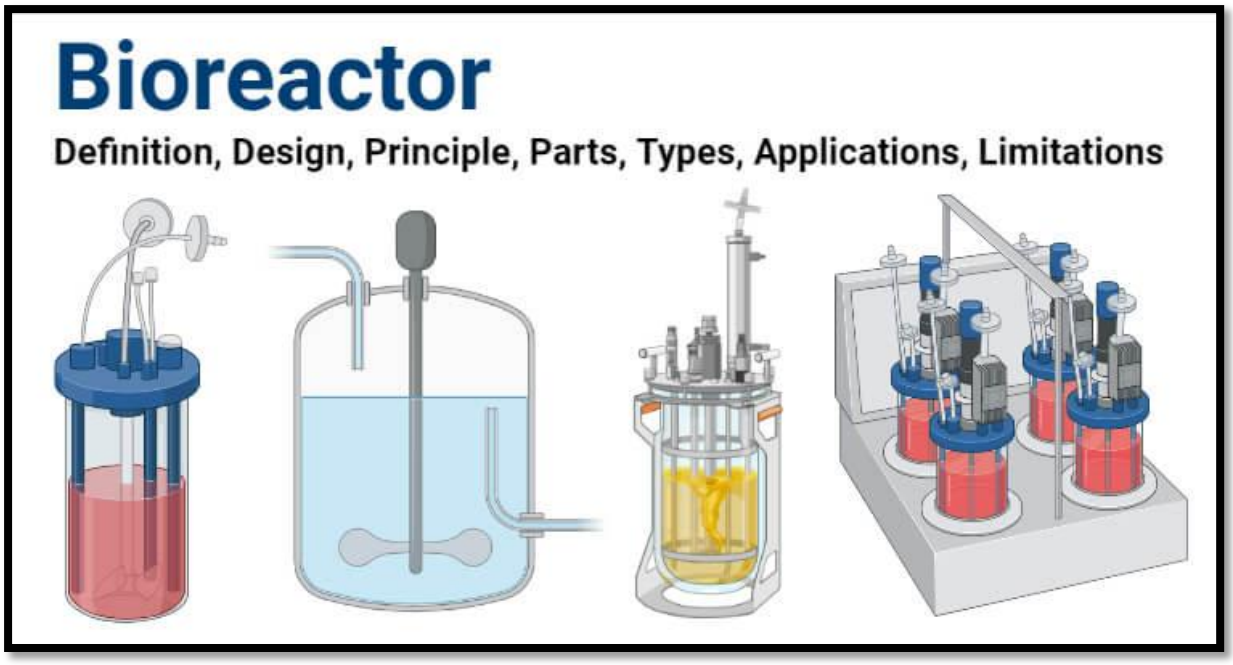
A bioreactor is a fermentation vessel that produces various chemicals and biological reactions.

It is a 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.

A bioreactor should provide for the following:

1. Agitation (for mixing of cells and medium),
2. Aeration (aerobic fermentors); for O2 supply,
3. Regulation of factors like temperature, pH, pressure, aeration, nutrient feeding, and liquid leveled.
4. Sterilization and maintenance of sterility, and
5. Withdrawal of cells/medium

Bioreactors are used for the production of biomass, metabolites, and antibiotics.



Bioreactor Design

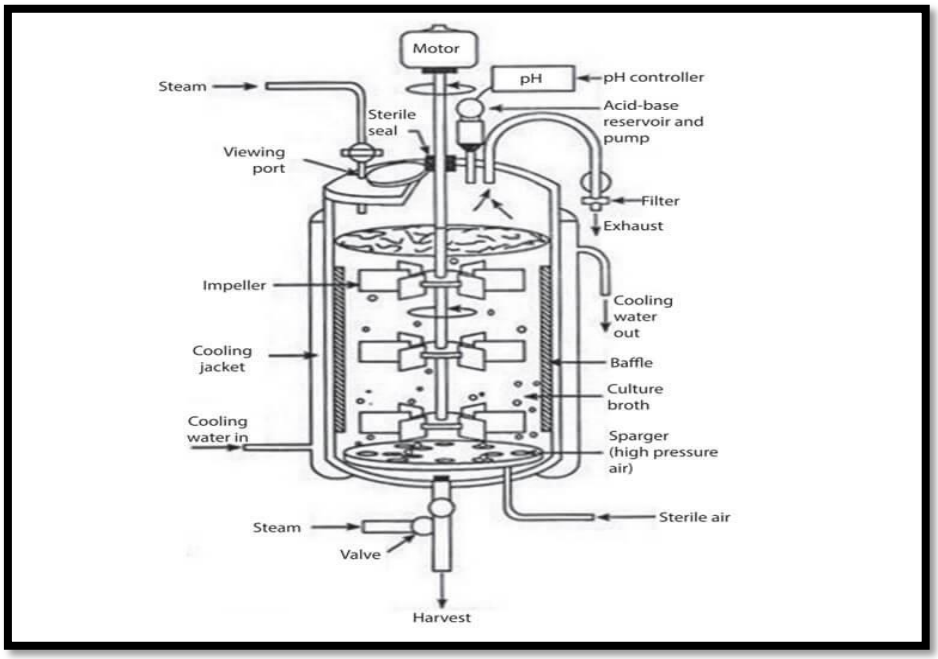
- The design and mode of operation of a bioreactor are based on the production of an organism, optimum conditions required for desired product formation, product value, and its scale of production.
- A good bioreactor design will help to improve productivity and provide higher quality products at lower prices.
- A bioreactor is a device that consists of various features such as an agitator system, an oxygen delivery system, a foam control system, and a variety of other systems such as temperature & pH control system, sampling ports, cleaning, and sterilization system, and lines for charging & emptying the reactor.
- The material used for the construction of a bioreactor must have the following important properties:
    - It should not be corrosive.
    - It should not add any toxic substances to the fermentation media.
    - It should tolerate the steam sterilization process.
    - It should be able to tolerate high pressure and resist pH changes.
- The sizes of the bioreactor vary widely depending on the application.
- Some bioreactors are designed for small scale fermenters and some for large scale industrial applications from the microbial cell (few mm3) to shake flask (100-1000 ml) to the laboratory-scale fermenter (1 – 50 L) to pilot level (0.3 – 10 m3) to plant scale (2 – 500 m3) for large volume).

Bioreactor Principle

- The bioreactor is the heart of any biochemical process as it provides an environment for microorganisms to obtain optimal growth and produce metabolites for the biotransformation and bioconversion of substrates into desirable products.
- The reactors can be engineered or manufactured based on the growth requirements of the organisms used.
- Reactors are machines that can be made to transform biological-based materials into desirable products.
- They can be used for the production of various enzymes and other bio-catalysis processes.

Parts of the bioreactor and their function

- These reactors have been designed to maintain certain parameters like flow rates, aeration, temperature, pH, foam control, and agitation rate.
- The number of parameters that can be monitored and controlled is limited by the number of sensors and control elements incorporated into a given bioreactor
- Other factors should be kept in mind before designing a fermenter as described below and demonstrated in the figure below.



1. Fermenter Vessel

- A fermenter is a large cylinder closed at the top and bottom connected with various pipes and valves.

- The vessel is designed in such a way that it allows to work under controlled conditions.
- Glass and stainless steels are two types of fermenter vessels used.
- The glass vessel is usually used in small-scale industries. It is non-toxic and corrosion-proof.
- Stainless steel vessel is used in large scale industries. It can resist pressure and corrosion.

2. Heating and Cooling Apparatus

- The fermentor vessel's exterior is fitted with a cooling jacket that seals the vessel and provides cooling water.
- Thermostatically controlled baths or internal coils are generally used to provide heat while silicone jackets are used to remove excess heat.
- A cooling jacket is necessary for sterilization of the nutrient medium and removal of the heat generated during fermentation in the fermentor.

3. Aeration System

- An aeration system is one of the very important parts of a fermentor.
- It is important to choose a good aeration system to ensure proper aeration and oxygen availability throughout the culture.
- It contains two separate aeration devices (sparger and impeller) to ensure proper aeration in a fermentor.
- The stirring accomplishes two things:
    - It helps to mix the gas bubbles through the liquid culture medium and
    - It helps to mix the microbial cells through the liquid culture medium which ensures the uniform access of microbial cells to the nutrients.

4. Sealing Assembly

- The sealing assembly is used for the sealing of the stirrer shaft to offer proper agitation.
- There are three types of sealing assembly in the fermenter:
    - Packed gland seal
    - Mechanical seal
    - Magnetic drives

5. Baffles

- The baffles are incorporated into fermenters to prevent a vortex improve aeration in the fermenters.
- It consists of metal strips attached radially to the wall.

6. Impeller

- Impellers are used to provide uniform suspension of microbial cells in different nutrient mediums.
- They are made up of impeller blades attached to a motor on the lid.
- Impeller blades play an important role in reducing the size of air bubbles and distribute them uniformly into the fermentation media.
- Variable impellers are used in the fermenters and are classified as follows.
    - Disc turbines
    - Variable pitch open turbine

7. Sparger

- A sparger is a system used for introducing sterile air to a fermentation vessel. It helps in providing proper aeration to the vessel.
- The sparger pipes contain small holes of about 5-10 mm, through which pressurized air is released.
- Three types of sparger are used
    - Porous sparger
    - Nozzle sparger
    - Combined sparger–agitator

8. Feed Ports

- They are used to add nutrients and acid/alkali to the fermentor.
- Feed ports are tubes made up of silicone.
- In-situ sterilization is performed before the removal or addition of the products.

9. Foam-Control

- The level of foam in the vessel must be minimized to avoid contamination, this is an important aspect of the fermentor.
- Foam is controlled by two units, foam sensing, and a control unit.
- A foam-controlling device is mounted on top of the fermentor, with an inlet into the fermentor.

10. Valves

- Valves are used in the fermentor to control the movement of liquid in the vessel.
- There are around five types of valves are used, that is,
    - globe valve,
    - butterfly valve,
    - a ball valve, and
    - diaphragm valve.

- A safety valve is built-in in the air and pipe layout to operate under pressure

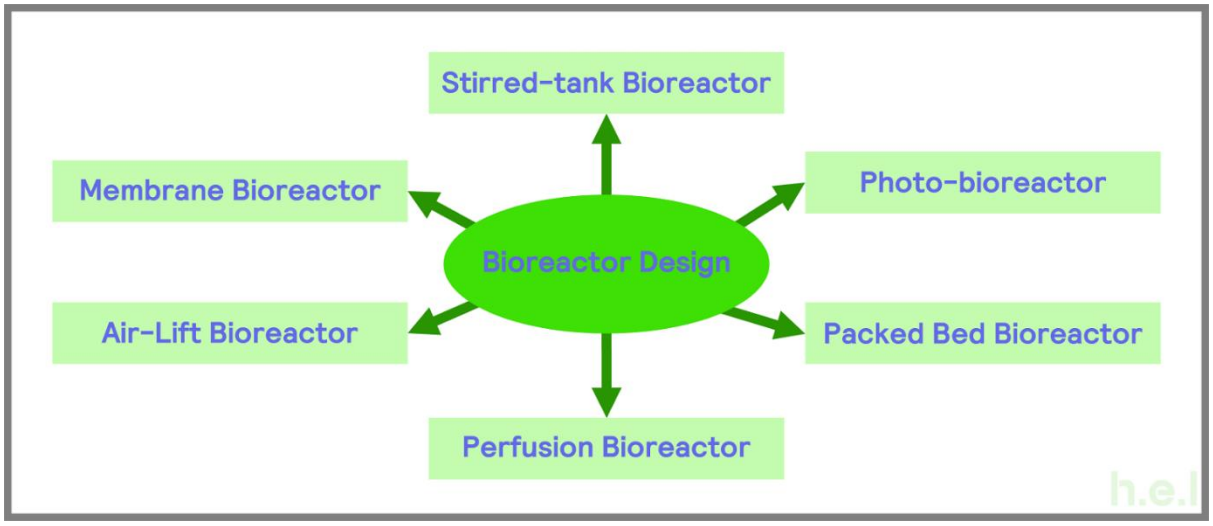
11. Controlling Devices for Environmental Factors

- A variety of devices are utilized to control environmental elements like temperature, oxygen concentration, pH, cell mass, essential nutrient levels, and product concentration.

12. Use of Computer in Fermenter

- For an efficient process, monitoring, and data collecting, fermentors are generally coupled with modern automated and semi-automated computers and databases.

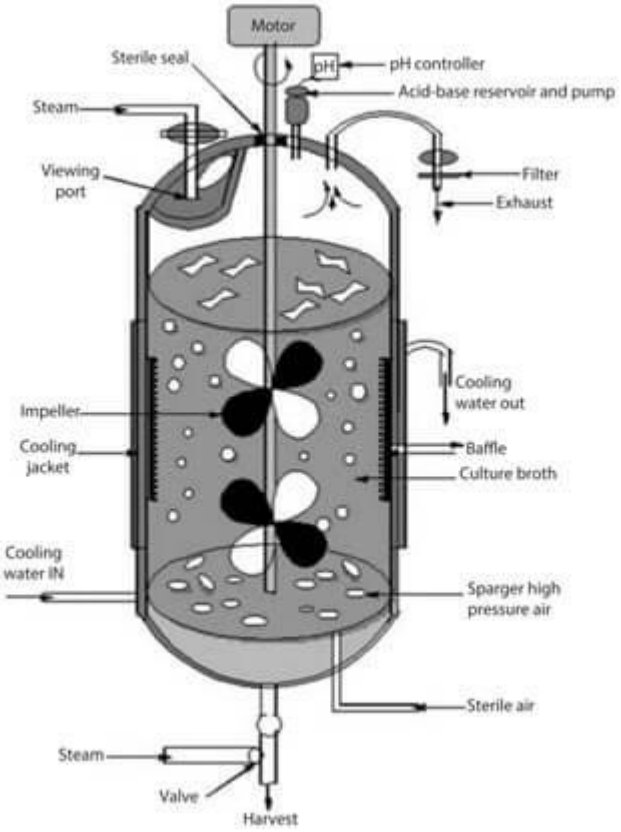
Types of bioreactor



The fermentor (bioreactor) types used extensively in industries are

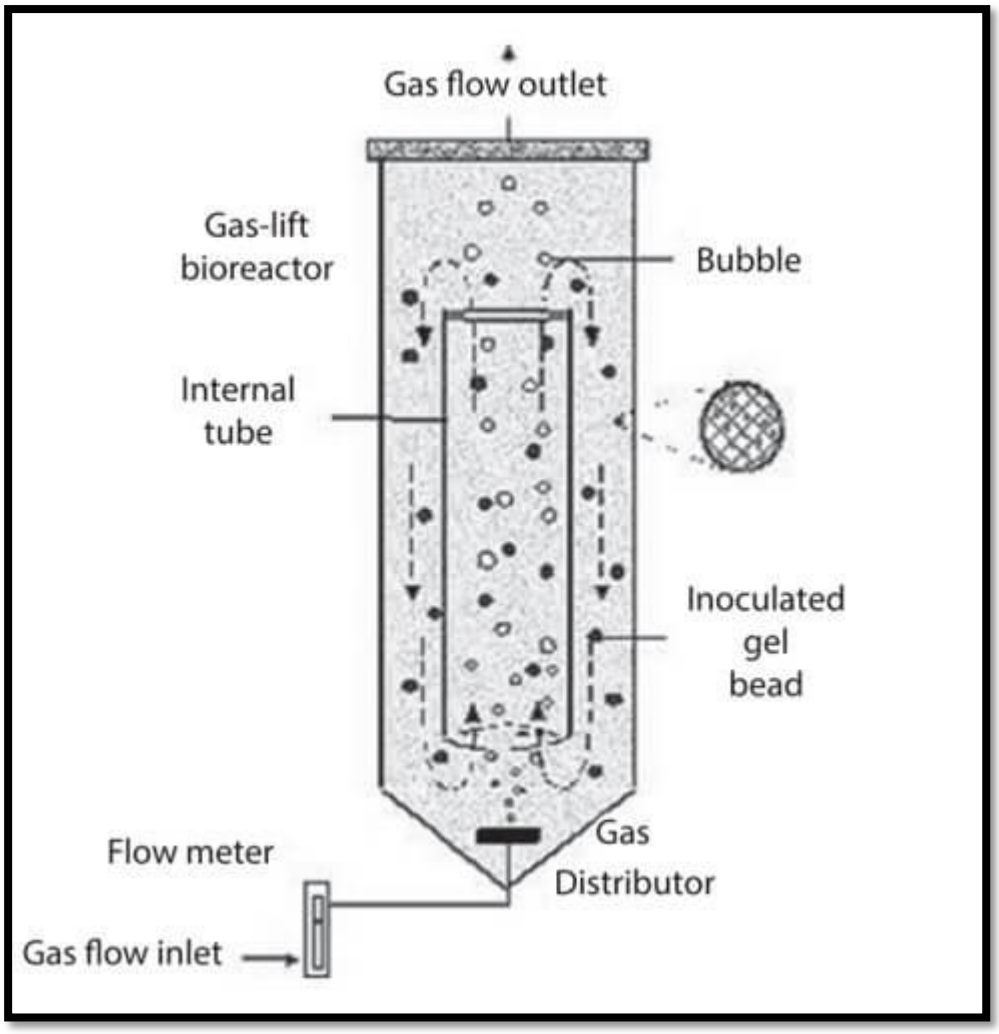
1. Continuous stirred tank fermentor

- A continuous stirred tank bioreactor is made up of a cylindrical vessel with a central shaft controlled by a motor that supports one or more agitators (impellers).
- The sparger, in combination with impellers (agitators), allows for improved gas distribution throughout the vessel.
- A stirred tank bioreactor can be operated continuously in the fermentor, temperature control is effortless, construction is cheap, easy to operate, resulting in low labor cost, and it is easy to clean.
- It is the most common type of bioreactor used in industry.



2. Airlift fermentor

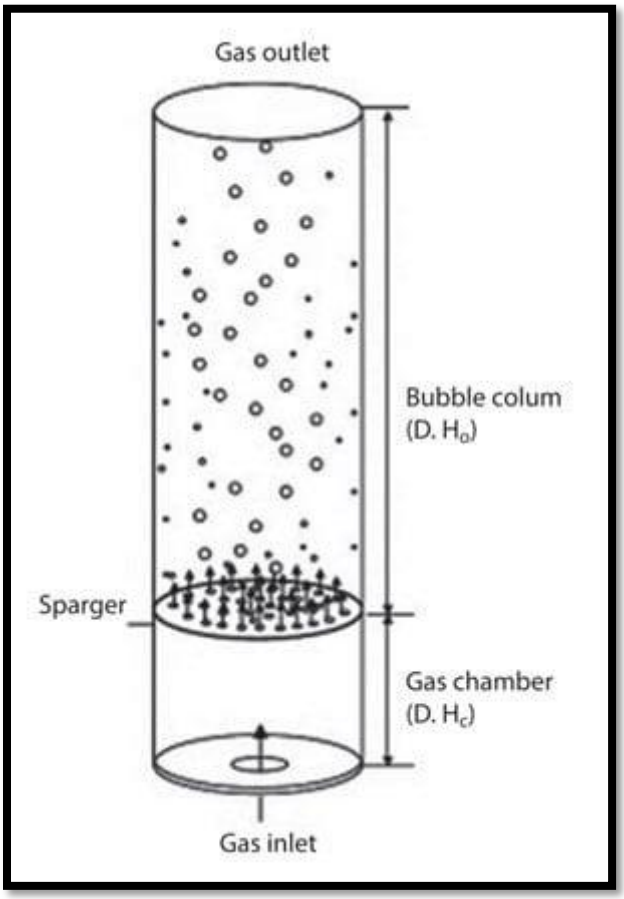
- The airlift reactor is generally used for gas-liquid or gas-liquid-solid contact devices. It is also known as a tower reactor.
- A bioreactor using an airlift system divides the fluid volume into two zones to improve circulation, oxygen transfer, and equalize forces in the reactor
- In a two-zone system, only one zone is sparged with gas. The zone where the gas is sparged is the riser; the zone in which it is not sparged in the downcomer.
- Airlift bioreactors are used for aerobic bioprocessing technology so that they can provide a controlled liquid flow in a recycling system using pumps.
- This equipment has several advantages such as its simplicity of design because it doesn't contain any moving parts or agitators, its easy sterilization, its low energy requirements, and its low cost.



3. Bubble column fermentor

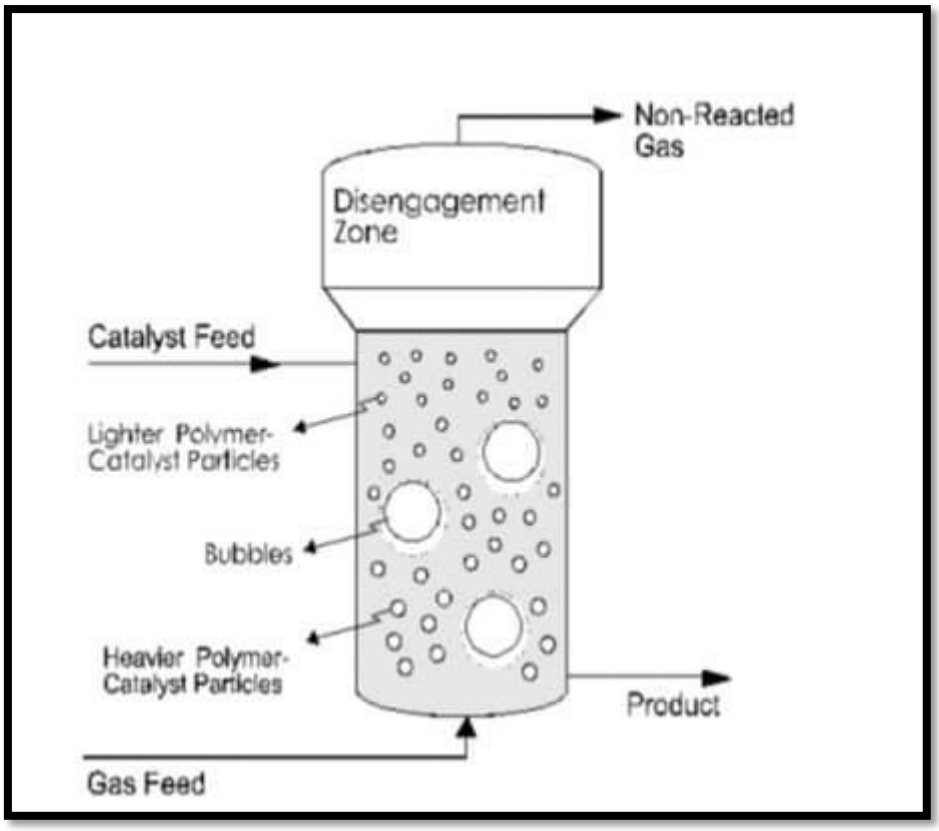
- The bubble column fermentor consists of a cylindrical vessel equipped with a gas sparger that pushes gas bubbles into a liquid phase or a liquid-solid suspension.
- The base of the column air or gas is introduced via perforated pipes or plates, or metal micro porous sparger.
- The rheological properties of the fluid and the gas flow rate have a significant influence on the mixing of O2 and other performance factors.
- To improve mass transfer and modify the basic design of the vessel, internal devices such as horizontal perforated plates, vertical baffles, and corrugated sheet packings placed are in the vessel.
- These reactors are simple in construction, easy maintenance, and have a low operating cost

- Bubble columns reactors are used in biochemical processes such as fermentation and biological wastewater treatment. It is also used in many chemical, petrochemical, and biochemical industries.



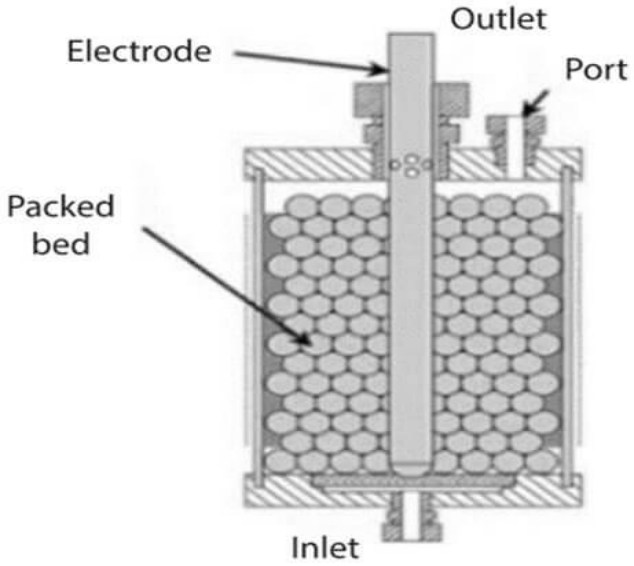
4. Fluidized-bed fermentor

- Fluid bed bioreactors constitute packed beds with smaller particles. This prevents problems such as clogging, high liquid pressure drop, channeling, and bed compaction associated with packed bed reactors.
- Catalyst is laid on the bottom of the reactor and the reactants are pumped into the reactor through a distributor pump to make the bed fluidized.
- In these reactors, the cells are immobilized small particles which move with the fluid as a result, mass transfer, oxygen transfer, and nutrition to the cells are enhanced.
- The bioreactors can be used for reactions involving fluid-suspended biocatalysts, such as immobilized enzymes, immobilized cells, and microbial flocs.
- Its main advantages include its ability to maintain even temperatures, easy replacement and regeneration of the catalyst, continuity, and automaticity of operation, and reduced contact time between gas and solid, compared to other catalytic reactors.



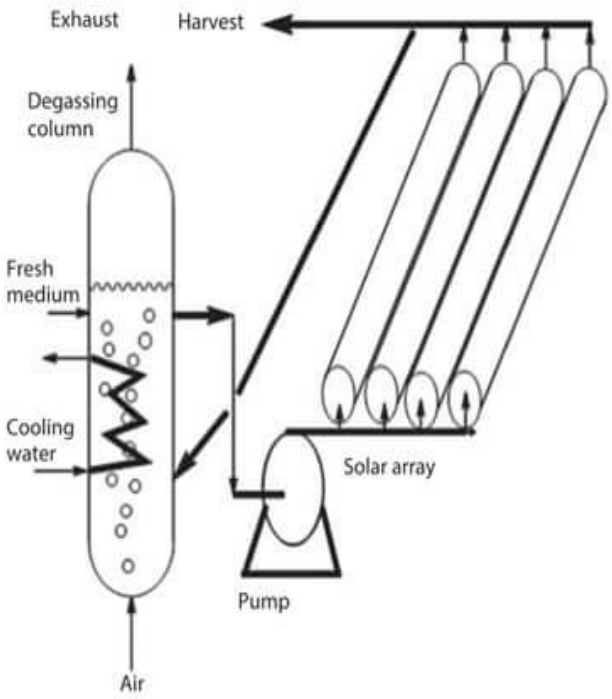
5. Packed bed fermentor

- A packed bed fermentor is a bed of solid particles, having biocatalyst on or within, the matrix of solids.
- It can either be run in the submerged mode (with or without aeration) or the trickle flow mode.
- Frequently used in chemical processing processes such as absorption, distillation, stripping, separation process, and catalytic reactions, packed bed reactors are also called fixed bed reactors.
- In packed-bed bioreactors, the air is introduced through a sieve that supports the substrate.
- This reactor has many benefits, like a high conversion rate for the catalyst, ease of operation, low construction and operation costs, increased contact between reactant and catalyst, and the ability to work in high temperatures and pressures.

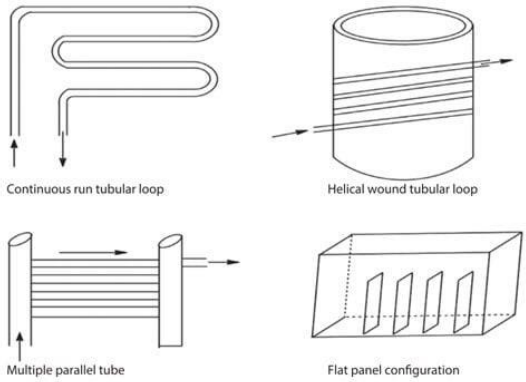


6. Photobioreactor

- A photobioreactor is a specialized unit for fermentation that is either illuminated by direct sunlight or artificially illuminated
- They are made up of glass or more commonly transparent plastic and the tubes or flat panels is consist of light receiving systems.
- In this bioreactor, centrifugal pumps or airlift pumps can be used to circulate the medium through solar receivers.
- Photo-bioreactors are usually operated in a continuous mode at a temperature in the range of 25–40 °C.
- Photobioreactors are used for the photosynthetic culture of microalgae and cyanobacteria to produce products such as astaxanthin and β-carotene.

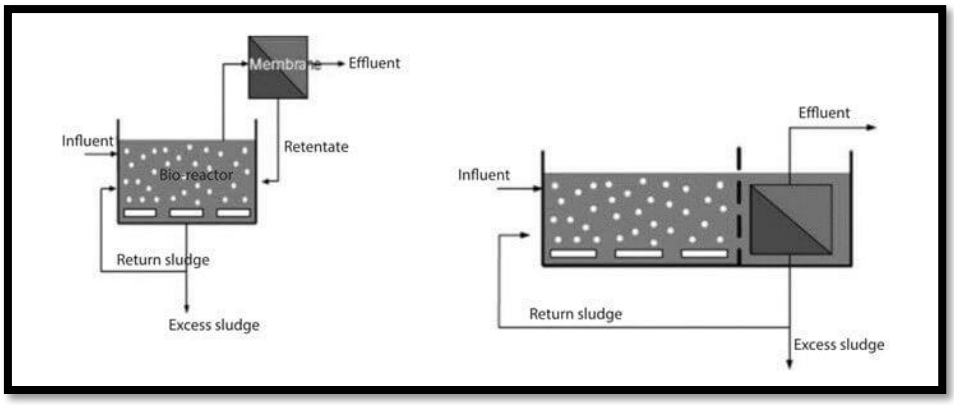


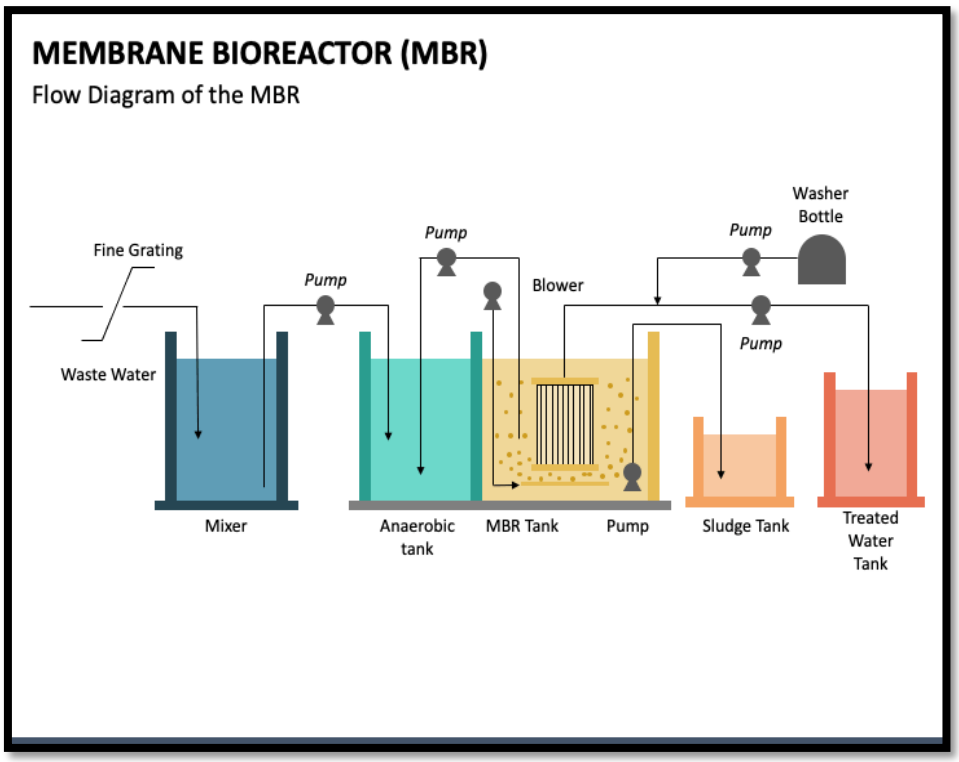
A tubular photobioreactor with parallel run horizontal tubes.



7. Membrane bioreactor

- This system combines traditional treatment with membrane filtration, resulting in the removal of organics and suspended solids as well as the removal of high nutrient levels.
- Membranes in this system are submerged in an aerated biological reactor. The pore size of the membrane ranges from 0.035 microns to 0.4 microns.
- With pure oxygen, the benefits of this bioreactor are enhanced resulting in even higher rate biological treatment systems that provide compact control of COD, microorganisms.





Applications of bioreactor

Some important applications of the bioreactor are:

Type of bioreactor	Applications
Stirred tank fermenter	Antibiotics, citric acid, Exopolysaccharides, cellulose, Chitinolytic enzymes, Laccase, Xylanase, Pectic, and pectate lyase, Tissue mass culture, Lipase, Polygalacturonases, Succinic acid
Bubble column fermentor	Algal culture, Chitinolytic enzymes

Airlift fermentor	Antibiotics, Chitinolytic enzymes, Exopolysaccharides, Gibberelic acid, Laccase, Cellulase, Lactic acid, Polygalacturonases, Tissue mass culture
Fluid bed fermentor	Laccase
Packed bed fermentor	Laccase, Hydrogen, Organic acids, Mammalian cells,
Photobioreactor	Wastewater treatment, water quality management, remediation of contaminated soil
Membrane bioreactor	Alginate, Antibiotic, Cellulose hydrolysis, Hydrogen production, Water treatment, VOCs treatment

Limitations of bioreactor

Types of bioreactor	Limitation
Stirred tank fermenter	High shear stress
	High power consumption
	Moving internal parts
Bubble column bioreactor	Low photosynthetic efficiency
Airlift	Non-uniform nutrient supply
	Insufficient mixing
	High viscosity can limit bulk circulation
Fluid bed fermentor	Particle (breakup) is common

	Increased reactor vessel size
	Bubbling beds of fine particles are difficult to predict and are less efficient.
	Pipe and vessel walls erode due to collisions by particles
Packed bed bioreactor	Undesired heat gradients
	Poor temperature control
	Difficult to replace the catalyst
Photobioreactor	Salability problems
	Require temperature maintenance as they lack evaporative cooling
	Periodic cleaning due to light exposure
	Need maximum light exposure
Membrane bioreactor	Biofilm overgrowth leads to periodic cleaning
	The membrane can rupture at high flow rates

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Citric Acid Production- Microbes, Methods, Steps, Factors

Citric acid (CA) has been widely produced as essential weak organic acid with extensive use in the pharmaceutical and food industries due to being a common metabolite found in animals and plants alike. Citric acid can be produced on a mass scale via synthetic or natural methods of extraction process or manufacturing the biomolecule. The natural process involves the extraction of citric acid from citrus plants like oranges, lime, lemons, etc. Whereas in the synthetic method, citric acid is produced by two approaches, i.e.

- The chemical approach of citric acid synthesis with the use of enzymes.
- A biological approach involving fermentation by the use of microorganisms like bacteria, yeast, etc.

Industrial production of CA using fermentation by microorganisms uses the 'submerged' fermentation technique where species like *Aspergillus niger* (fungi) and *Candida* (yeast) metabolize various carbon sources such as molasses and starch-based culture media. Other fermenting techniques like surface fermentation and solid-state fermentation take use of agro-based residues as the carbon source for the mass production of citric acid.

Properties of Citric Acid

- The citric acid (CA) is a naturally occurring weak acid with a molecular formula $C_6H_8O_7$ and IUPAC molecular name being '2-hydroxy-1,2,3 propane tricarboxylic acid'.
- This tricarboxylic acid has a molecular weight of 210.14 g/mol.
- They naturally occur in citrus plants like lemon and also is a commonly found metabolite in animals.
- Citric acid is a common primary metabolite derived from carbohydrates, proteins, and fats of the Krebs (tricarboxylic) cycle for energy needs.
- The melting point of CA is 153 degrees Celsius.
- When CA is in the pure state, it is soluble in water and other polar solvents like acetone, ethanol, etc.
- At room temperature, citric acid is present in the solid state.
- Produced massively via chemical (enzymatic) biosynthesis or biologically by the microbial process of fermentation.

Historical Events

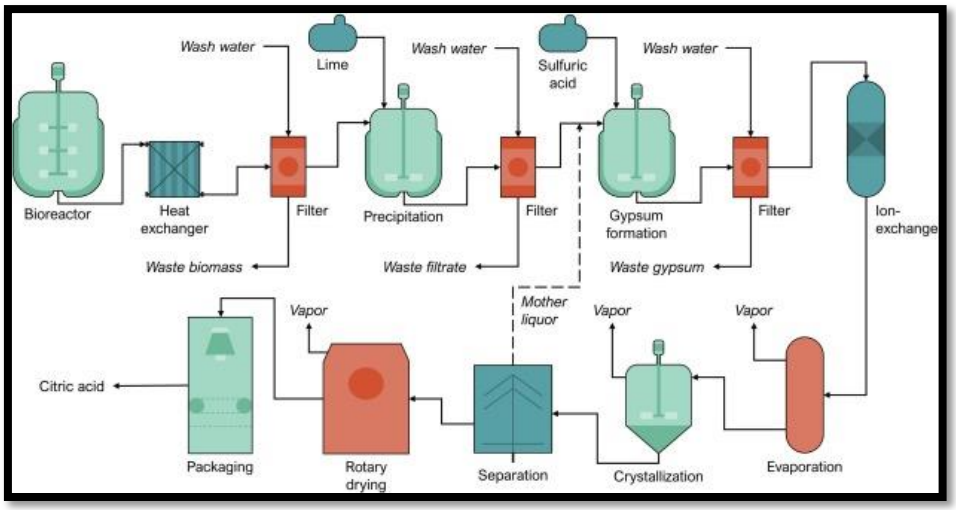
- **Karl Scheels** in the year **1874,** was the first scientist to isolate citric acid by crystallization process from lemon juice.

- In **1880,** two scientists named, **Adams and Grimaux** synthesized citric acid from glycerol.
- In **1893**, **Wehmer** tested fermentation carried out by the Penicillum gaucum species and observed the accumulation of CA as a by-product of calcium oxalate.
- In **1917, James Curie** a food chemist demonstrated that Aspergillus niger yielded a larger amount of citric acid than other organisms at the time in a sugar-based medium.
- **Millard in 1922**, accumulated citric acid by limiting nutrients in the bioreactors where fermentation was carried out by Aspergillus niger.
- By the year **1965**, yeasts were introduced along with alkanes and carbohydrates as substrates.
- In **1984,** the **'Submerged fermentation technique**' was introduced to produce large quantities of citric acid in the US industries.

Citric Acid Biochemistry and Accumulation

In 1953, Hans Adolf Krebs was awarded Nobel Prize in Physiology or Medicine for the discovery of the Citric Acid cycle. Citrate or Citric acid is an intermediate metabolite in the Krebs cycle (also known as Tricarboxylic acid or Citric acid cycle) found in bacteria, plants, and animals. To produce citrate, the condensation of acetyl CoA to oxaloacetate is catalyzed by the citrate synthase enzyme. Citrate acts as a substrate for the downstream reaction of the TCA cycle and the cycle ends with the renewal of oxaloacetate. This citrate is exported to the cytoplasm from mitochondria to serve as a substrate and regulate various biochemical synthesis of biomolecules.

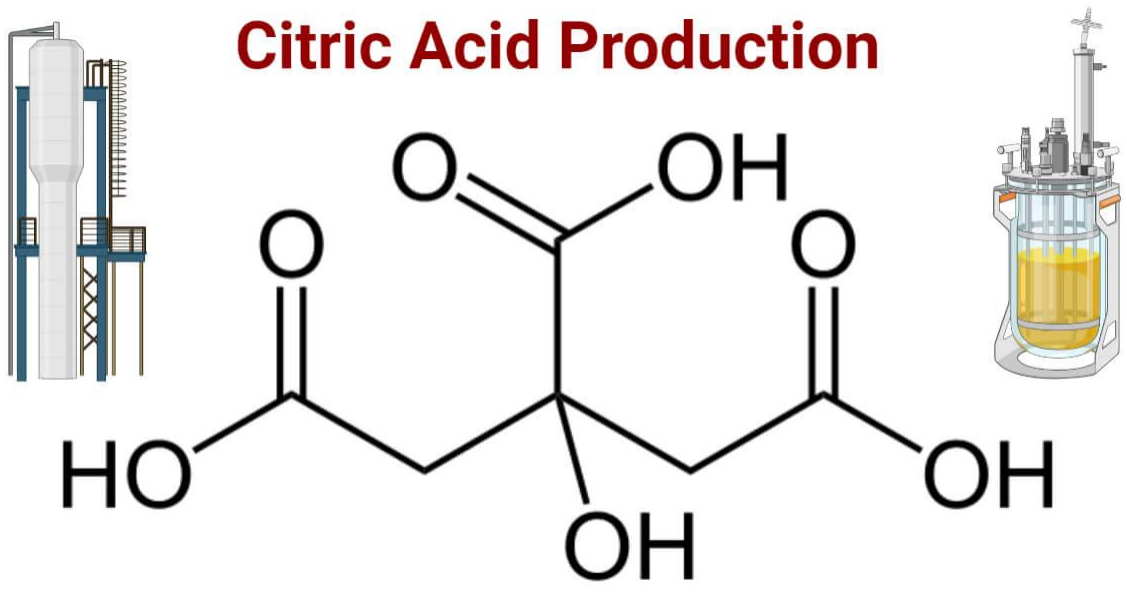
It suggested that enzymes responsible for the degradation of CA in the Krebs cycle must be deactivated to accumulate Citric Acid. But it is also observed that the Krebs cycle is active for biomass production. Another hypothesis is that tricarboxylate transporter activity competes with aconitase for binding to CA. The transporter has a higher affinity for CA where it ejects the citrate out of mitochondria to cytoplasm without inhibiting kreb cycle enzymes.



Microorganisms Used in CA Production

Aspergillus niger is the highly recommended industrial producer of citric acid. They flourish in sugar medium containing salts at pH 2.5-3.5 and excrete large volumes of CA. An estimated practical yield of CA is 70 % of the theoretical estimate which is 112g per 100g of sucrose.

Many organisms like ***Penicillin sp., Eupenicillin sp., Botrytis sp., Absidia sp., Ustulina vulgaris,*** and more can also accumulate citric acid in a sugar and inorganic salt medium. Yeast species belonging to the genera of ***Hansenula, Candida, Torula, Saccharomyces, Pichia,*** etc, can produce CA from carbohydrates and n-alkanes. However, the production of CA via these organisms may not seem economical due to the accumulation of unwanted by-products like isocitric acid. A remedial approach could be the development of a mutant strain with lower aconitase activity.



Industrial Citric Acid Production Approach

An estimated 99% of CA mass production worldwide is carried out by microbial processes, surface or submerged culture methods. The final product obtained is marketed as a monohydrate acid or an anhydrous salt. About 70% of total CA is used in the beverages and food industries as an acidifier or antioxidant for the preservation and enhancement of flavors, and aromas in ice cream, marmalades, and fruit juices. 20% of total CA produced is utilized by pharmaceutical industries as blood preservatives, vitamin preservant as antioxidants, effervescent, and iron citrate in cosmetics, tablets, and ointments. In the chemical industry, it is used as a foaming agent for softening textiles and a phosphate substitute for hardening cement.

a. Surface Culture

Surface culture is divided into solids and liquids, though the liquid approach provides higher economic production.

- The culture medium is put on a shallow aluminium tray 5-20 cm deep and maintained at 5-6 pH.
- Uniform air circulation and maintenance of temperature and humidity are ensured in the fermentation chamber.
- Then *A. niger* spores are blown onto the culture medium surface for 5-6 days with uniform passage of dry air.
- The pH of the medium is changed to 1.5 to 2.
- After 24 hours of incubation, the spores germinate and the white mycelium forms on the medium surface.
- The moulds start to utilize the sugar content of the medium and after complete utilization, the residual liquid is extracted from the mycelium mesh.
- This residual liquid contains CA and *A. niger* produces a small volume of CA as the main metabolite in this method.

Inoculum Preparation

- A suitable and high-yielding *A.niger* strain is selected from the stock culture.
- Glass vials that are incubated for 10-14 days and then sporulating media are infected with the selected stock culture.
- In the sporulating media, trace elements like zinc, iron salts, and manganese should be maintained.
- To suspend the mature spores, the appropriate diluent is used like water with the wetting chemical **sodium lauryl phosphate**.

Media Preparation

- For CA production, the medium must contain a carbon source such as sucrose and inorganic salts.
- **Sucrose and beet molasses** are preferred carbon sources for CA fermentation media.
- To achieve a high CA yield, a culture medium with less than 15% sucrose is beneficial.
- The use of fructose or glucose instead of sucrose leads to lower citric acid output.
- On a commercial scale, beet molasses are the preferred carbon source for A. niger.
- Beet molasses are rich in inorganic salts, hence they are pre-treated with ferricyanide or ferrocyanide to diminish excess inorganic salt.
- Other elements added to the medium for optimum growth of microorganisms and CA production are nitrogen, phosphorus, potassium, and magnesium.
- Salts like ammonium nitrate, magnesium sulfate, potassium mono hydrogen phosphate, etc, are also added in small amounts.
- pH is adjusted by the addition of HCl to 3.4 – 3.5 as lower pH facilitates sterilization, less contamination, suppression of oxalic acid formation, and good yield of citric acid synthesis.

Fermentation

- The prepared media of 1 to 2.5 cm thick is placed in a shallow pan.
- The inoculum spores are spread onto the medium surface with uniform aeration to allow even distribution of spores in the media.
- Incubation is done at 30-40 degree celsius temperature.
- The temperature is held constant during fermentation with air current ventilation to allow efficient gaseous exchange.
- After 24 hours of inoculation, the germinating spores produce a thin layer of mycelium on the medium surface.
- the pH of the medium drops to 1.5 – 2.0 as ammonium ions are formed.
- Presence of oxalic acid and yellow pigment after 30 hours of fermentation is an indication of high iron concentration and halted citric acid production.
- Fermentation ceases after 8-14 days with thick mature white mycelium floating atop of liquid media.
- The ratio of surface area to medium volume is proportional to the rate of sugar conversion to CA.
- In the shallow pan method, the ratio is reduced and the yield of CA increases.
- Under appropriate conditions, sugar is exponentially converted to citric acid.

- This method yields between 1.2 and 1.5 kilogrammes of citric acid monohydrate per square metre of fermentation surface per hour.

Recovery

- Mycelium is separated from the mycelium broth.
- Mycelium is pressed to extract any intracellular citric acid present.
- The filtered broth is treated with Calcium hydroxide and then treated with sulphuric acid in equivalent volume to obtain CA and calcium sulfate is generated as a precipitate.
- Filtration is employed to isolate precipitate, and impure citric acid is generated by demineralizing and decolorizing it with activated carbon.
- After evaporation, pure CA crystals are obtained.

b. Submerged Culture

This fermentation approach recruits ***Aspergillus japonicus***, a black aspergillus. Submerged culture is done in stainless steel bioreactors with a cooling system, aeration, impeller, etc, equipment. Carbon source substrates include maize starch, beet molasses, etc, and for nitrogen supplements, ammonia is utilized. The culture medium is maintained at 30 degrees celsius when A. japonicus is added to the culture. The use of batch bioreactors generates 1500 kg of CA and 500 kg of biomass from using 2500 kg of glucose and 860 kg of O2. The quality of the metal recruited to produce fermenters, mycelium structures, and oxygen delivery systems, greatly influences the production rate. An alkane metabolizing fungus, *Candida lypolytica* is employed in a continuous fermentation approach for citric acid production, yielding 45% more CA than standard procedure.

Inoculum Preparation

- A suitable and high-yielding *A. japonicus* strain is extracted from the stock culture.
- As an inoculum for fermentation, mycelial mats are used known as pellets.
- In the seed fermenter, 15% molasses sugar is used to induce spore germination.
- To stimulate high mycelial production, the appropriate concentration of cyanide ions is introduced to the medium.
- **To avoid regular mycelium production which is seen in the case of lower cyanide concentration, high cyanide concentration is required for mycelial pellet formation.**
- In 24 hours and at 32 degrees Celsius, the spores germinate forming 0.2 to 0.5 mm pellets.
- The pH drops to 4.3, and pellets are used as a starter culture for production fermenters.

Media Preparation

Same preparatory protocol as the surface media culture is made.

Fermentation

- Stainless steel fermenters are used to avoid heavy metal leaching.
- Mycelial pellets that are grown in a seed fermenter are transferred to the steel production fermenter with a minimum holding capacity of 1000 litres and maintained at 30 degrees Celsius temperature.
- The loose and few branched filamentous mycelia with no chlamydospores are observed to produce lower citric acid.
- The iron-to-copper ratio is essential for mycelial growth and induces pellet form, which causes optimum CA production.
- The optimum oxygen concentration for high CA yield is 20 – 25 % of the saturation value during fermentation.
- The aeration rate should be maintained at 0.2 – 1 volume per minute during the acid production phase.
- **To avoid foaming issues, lard oil, an antifoaming agent is utilized.**

Recovery

Similar protocols as applied in the surface culture media.

Limiting Factors during Citric Acid Production

Fungal Species

Many fungi species such as ***A. niger, Penicillium luteum, Ustulina vulgaris,*** etc., have been demonstrated to produce citric acid, hence it becomes crucial to select quality and high-yield strains for optimum CA production. *A. niger* is the widely preferred strain because they are highly productive, simple to cultivate, and produce a very low amount of unnecessary by-products like oxalic acid.

Carbon Source

To produce massive amounts of quality citric acid, sugars that are easily assimilated and metabolized by the microorganisms used. If the microbe produces hydrolytic enzymes effective at the lower pH required for fermentation, polysaccharides can be a useful raw material. **Sucrose is preferred over glucose** as a carbon source because A. niger possesses an invertase enzyme in the mycelium which catalyzes the hydrolysis of sucrose into glucose and fructose.

Widely utilized industrial carbon sources are **sugar beet molasses, lower-quality sugarcane byproducts with levels of cations, and glucose syrup from starch hydrolysis**. The highest productivity is seen in the medium containing 15 – 22% sugar.

Nitrogen and Phosphate

Complex media containing **beet molasses are rich in nitrogen** and laboratory-prepared media are supplemented with **ammonium sulfate and nitrates**, which favours lower pH essential for fermentation. **Urea** can also be used as a nitrogen source for media preparation. The use of phosphate promotes biomass growth due to enhanced secondary reactions.

Culture Medium pH

There are two stages of pH fluctuation seen. For spore germination, the pH of the medium should be lower than 5. And during the production phase, the pH drops to 2 or less due to the absorption of ammonium ions by germinating spores which leads to the release of protons, aiding in citric acid production. Lower pH also decreases the chance of pathogen contamination and inhibits the production of undesirable organic acids like oxalic and gluconic acids.

Aeration

Fluctuations in the aeration can have a detrimental impact on CA production. Lower levels of CO2 are caused due to high aeration rate. CO2 is an important substrate for pyruvate carboxylase enzyme that replenishes oxaloacetate for citrate synthase affecting CA output. Higher levels of carbon dioxide are also not beneficial for optimum citrate biomass.

Trace Elements

Balanced levels of trace elements Mn, Fe, Zn, Cu, etc, are necessary for fungal growth and high CA output. Levels to maintain for :

- Zinc – 0.3 ppm
- Iron – 1.3 ppm
- Manganese – 3 mg/l

Manganese regulates many cellular functions like sporulation, cell wall synthesis, and the production of secondary metabolites.

Conclusion

Citric acid or CA is a weak organic acid commonly found in all organism and commercially they are used as a preservative, acidulant, and flavouring agent in beverages, foods, cosmetics, and pharmaceutical drugs. Produced industrially via fermentation approach by microorganisms of fungal and bacterial species like Aspergillus niger, Escherichia coli, etc.

The fermentation process involves several regulated steps like sterilization, inoculation, media preparation, fermentation, recovery, and downstream processes. The sterilized substrate (any carbon source like molasses, sucrose, etc.) is inoculated with CA-producing microorganisms in a culture medium. The inoculated substrate is transferred to a bioreactor where further fermentation is achieved under optimum conditions of temperature, aeration, and pH.

Microorganism metabolizes substrate molecules and releases citric acid as a by-product. After the completion of the fermentation process, the fermented broth is sent for recovery and purification of CA which involves steps like filtration, crystallization, and drying to form anhydrous white crystals to be sold for commercial purposes.

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Human insulin production by genetic engineering

- **Insulin** is a hormone produced by β-cells of islets of Langerhans of pancreas. It was discovered by sir Edward Sharpey Schafer (1916) while studying Islets of Langerhans.
- Pancreas is a mixed gland situated transversely across the upper abdomen behind stomach and spleen.
- Insulin is a peptide hormone produced by pancreas and is a central regulator of carbohydrates and fat metabolism in the body.

Structure of Human Insulin:

- Chemically Human insulin is small, simple protein composed of 51 amino acids sequences and has a molecular weight of 5808 Da.
- Insulin hormone is a dimer of a A- chain and a B-chain which are linked together by a disulphide bond.
- Fredrick Sanger et al (1954) gave the first complete description of insulin. Insulin consists of two polypeptide chain,
  - Chain A- 21 amino acids long
  - Chain B-30 amino acids long
  - Both chains are joined together by disulphide bond between two cysteine residue

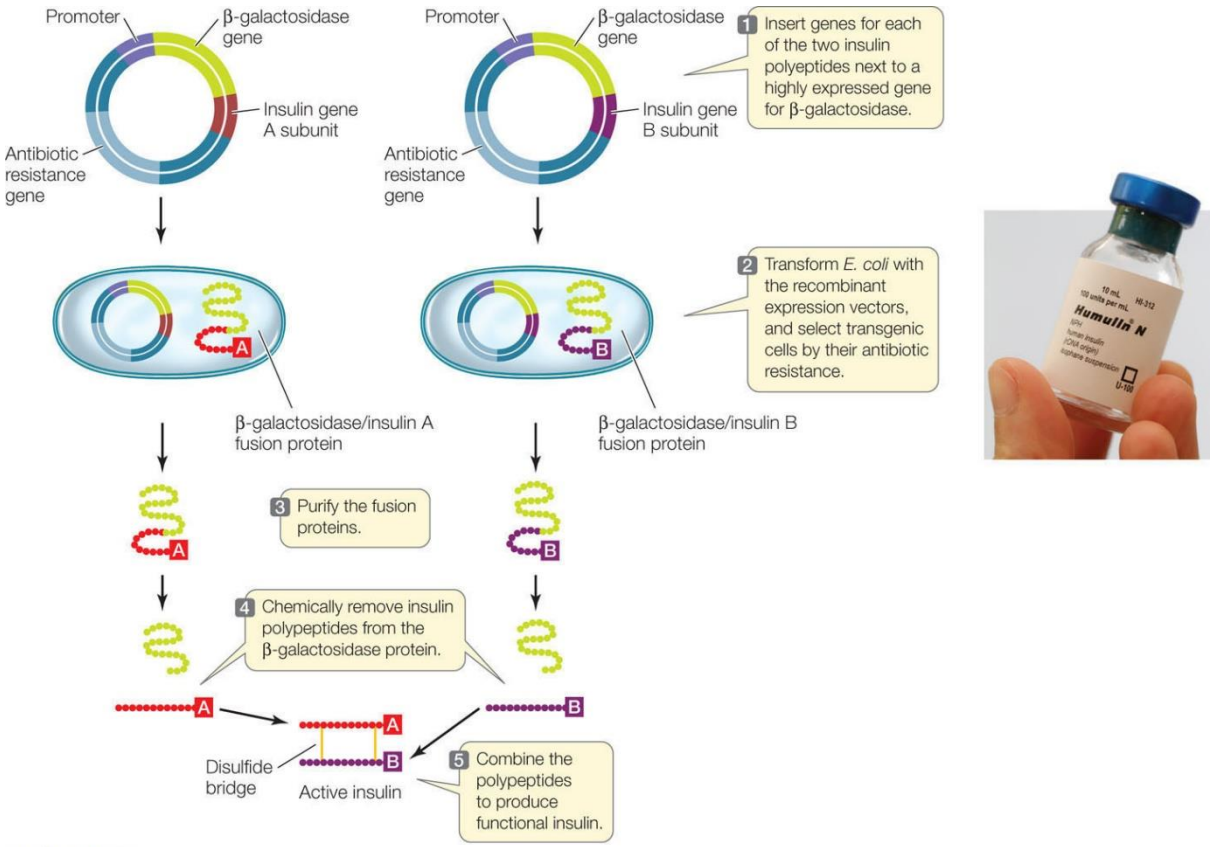
Insulin produced inside pancreas:

- At first Pancreatic β-cells synthesize pre-pro-insulin, which is a 109 amino acids long polypeptide
- Among 109 amino acids, 23 amino acids are signal molecules which allows the pre-pro-insulin to pass through cell membrane.

- Entering inside cell, it become 86 amino acids long pro-insulin. It is still inactive.
- Some Proteolytic enzymes cut and expose the active site of pro insulin converting it into active form of insuin of 51 amino acids long.

Insulin produced by recombinant DNA technology.

- The basic step in recombinant DNA technology is similar for insulin production also.
    - At first suitable vector (plasmid) is isolated from E. coli and then it is cut open by restriction endonuclease enzyme.
    - The gene of interest (ie. Insulin coding gene) is isolated from β-cell and inserted in opened plasmid.
    - Plasmid and gene of interest are recombined together by DNA ligase enzyme
    - This recombined plasmid is inserted into suitable host cell (ie E. coli) and now this recombined host cell starts producing insulin hormone.
- Hakura et al (1977) chemically synthesize DNA sequence of insulin for two chains A and B and separately inserted into two PBR322 plasmid vector.
    - These gene are inserted by the side of β-galactosidase gene of the plasmid.
    - The recombinant plasmid were then separately transformed into E. coli host.
    - The recombinant host produced pro-insulin chains ie. fused β-galactosidase-A chain and β-galactosidase-B-chain separately.
    - These pro-insulin chains A and B were separated from β-galactosidase by treatment with cyanogen bromide. The detachment of pro-insulin chains from β-galactosidase is possible because an extra codon form methionine was added at N-terminal of each gene for A and B-chain.
    - After detachment, A and B chains are joined invitro to reconstitute the naïve insulin by sulphonating the peptide chains with sodium disulphonate and sodium sulphite.



figure; diagrammatic representation of insulin production by genetic engineering

- Another method of insulin production by recombinant DNA technology is designed by Gilbert and Villokomaroff.
    - In this method, m RNA for pre-pro-insulin is isolated from islets of Langerhans cell
    - mRNA is reverse transcribed to form DNA and then it is inserted into PBR 322 plasmid in the middle of the gene for penicillinase.
    - Then the recombinant plasmid is transformed into suitable host ie E. coli cell
    - The host produced penicillinase + pre-pro insulin
    - Insulin is later separated by trypsin treatment

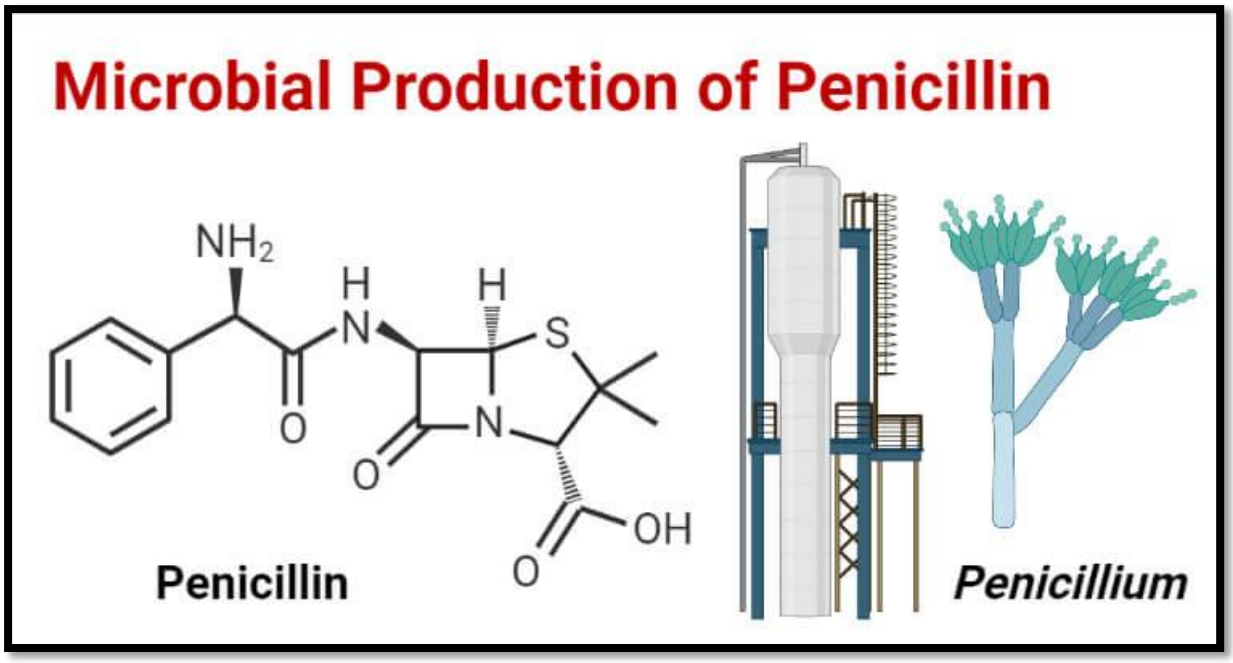
Roles of insulin in body:

1. Insulin causes cells in liver, skeletal muscles and fat tissue to take up glucose from the blood. In liver and skeletal muscle, glucose is stored as glycogen and in adipose tissue, it is stored as triglyceride.
2. Insulin stops the use of fat as energy source by inhibiting the release of glucagon hormone.

3. With the exception of the metabolic disorder such as Diabetes mellitus and metabolic syndrome, insulin maintain constant proportion of glucose in blood by removing excess glucose from the blood which otherwise would be toxic.
4. When blood glucose levels fall below a certain level, body begins to use stored glycogen as energy source through glycogenolysis; which breaks down glycogen stored in liver and muscles into glucose, which is then utilized as energy source.
5. Failing to control the level of insulin in body results in a disorder called diabetes mellitus. As a consequences Insulin is used medically to treat some forms of diabetes mellitus.
6. Patients with type I diabetes depends on insulin shots. Most commonly insulin is injected subcutaneously for the patients because the hormone is no longer produced in their body. Type I diabetes is also known as Insulin dependent diabetes mellitus.
7. Patients with type II diabetes are often resistant to insulin and because of such resistance many suffer from relative insulin deficiency. This is also known as Insulin independent diabetes. Some patients with type II diabetes may eventually require insulin shots if other medication fails to control blood glucose level. Over 40% of type II diabetes patients require insulin shots as part of their diabetes management plan.

Penicillin Production

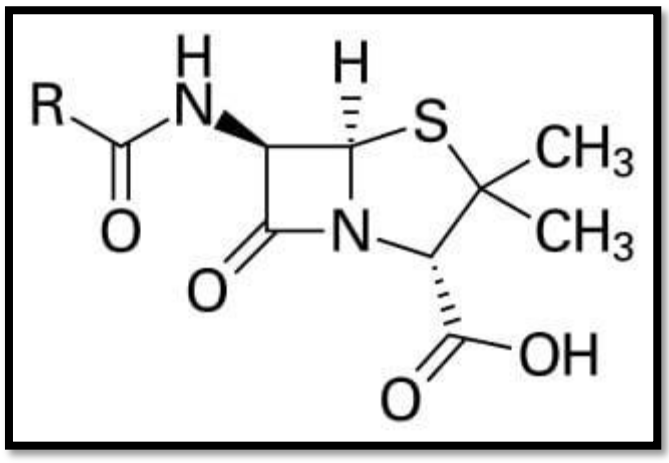
Alexander Fleming in September 1928 accidentally discovered Penicillin. He found that the fungus, *Penicillium notatum* prevented the growth of bacteria, *Staphylococcus* spp.. Later Clutterbuck and his colleagues in 1932 studied the nature of Penicillin and found it as an organic acid that dissolves into the organic solvent at low PH. Chain et al in 1940 cultured fungus and extracted powdered form of Penicillin. Later, at the time of the second world war, Penicillin production was done and an adequate amount was produced to treat wounded people. *P. notatum* gave poor results so, other species of *Penicillium* were tested. As compared, *P. chrysogenum* NRRL 1951 gave good results which were induced by UV and other mutagenic chemicals. These selected strains produced a huge amount of Penicillin and inhibit the growth of the Oxford strain of *Staphylococcus aureus*. Czapek-Dox broth was used for the culture of *P. notatum*. Later, casein, beef extract were added for the better yield of penicillin which will be an aid in production. In 1949 chemically produced mediums like phenylacetic acid ethyl amine etc by maintaining the PH and addition of buffering agents like Calcium carbonate and also maintain the temperature.



Types of Penicillin

Two types of Penicillin are Penicillin G (Benzyl Penicillin) and Penicillin F. Penicillin F is also known as Phentenyl Penicillin. Natural Penicillin are obtained as sodium or potassium salts. These classes of antibiotics are used in treating both Gram-positive and Gram-negative infections. Penicillin G are a narrow-spectrum antibiotic. Examples of Penicillins are Ampicillin, Cloxacillin, Oxacillin, Piperacillin, etc.

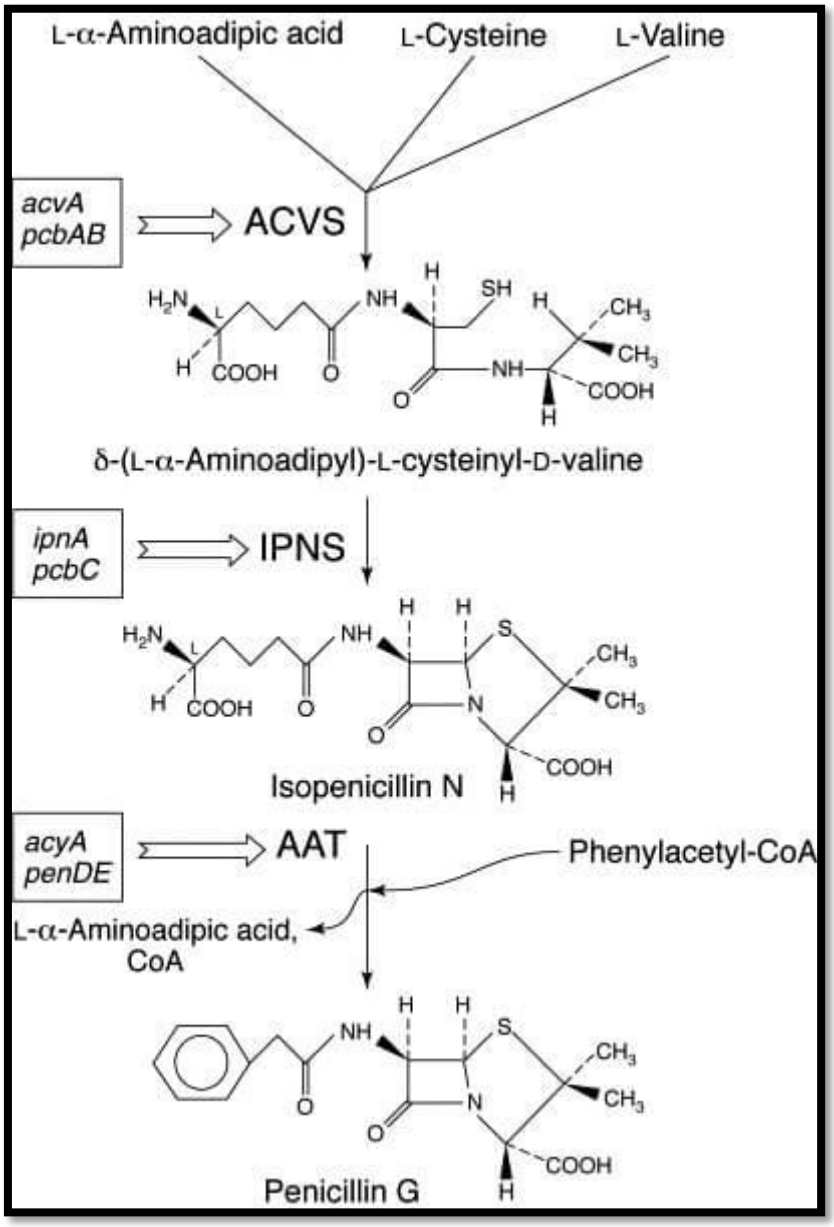
Structure of Penicillin



The structure of Penicillin includes a 4-membered β-lactam ring and thiazolidine ring. B-lactam ring contains an amide bond that is broken in an acidic and alkaline medium and that bond is hydrolyzed by beta-lactamase which is synthesized by many bacteria. Naturally occurring penicillins have different structure which is separated by R groups. The basic structure attaches

to the N-acyl group in the substituted amino group. Mainly, Penicillin is categorized as natural and semisynthetic.

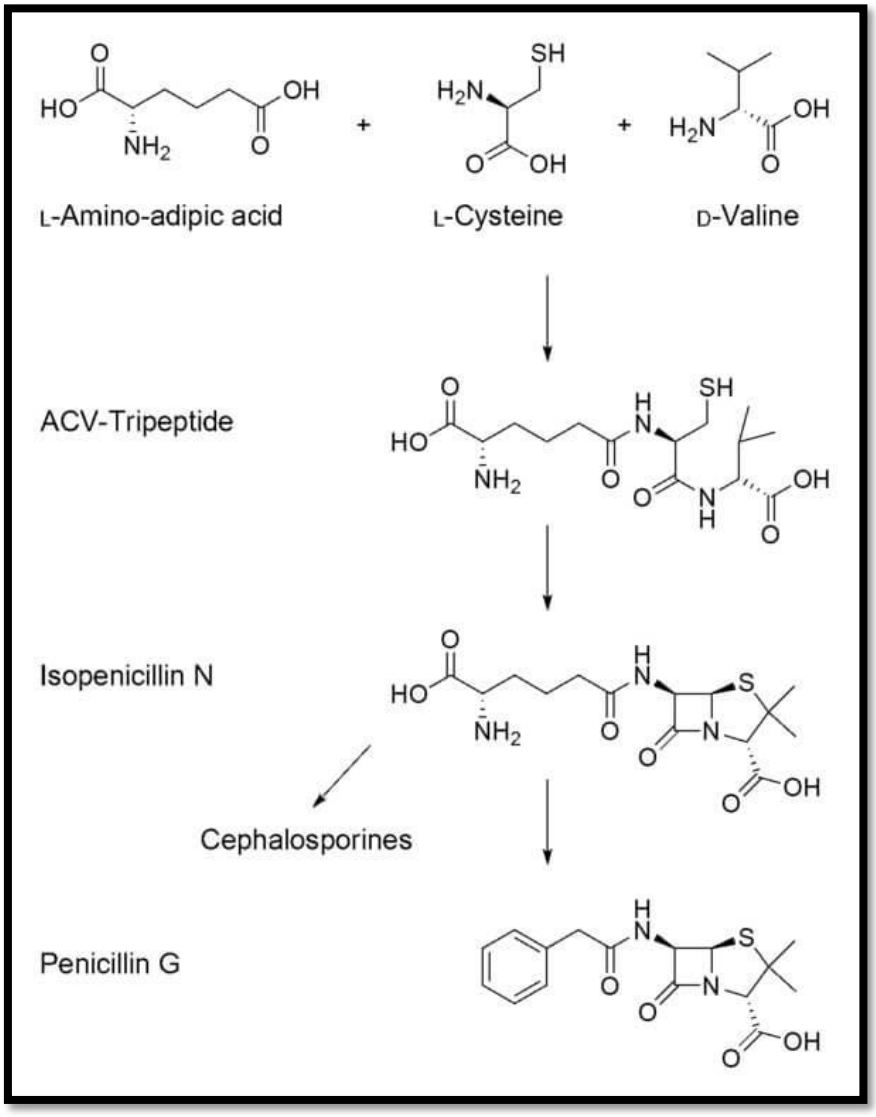
Biosynthesis of Penicillin



Penicillins yield is done commercially by using *P.chrysogenum*. Although the fungus was found earlier in 1928, these biosynthesis processes were concluded later. Penicillin biosynthesis is described into three main steps; catalytic step, oxidative, and exchange of different chains.

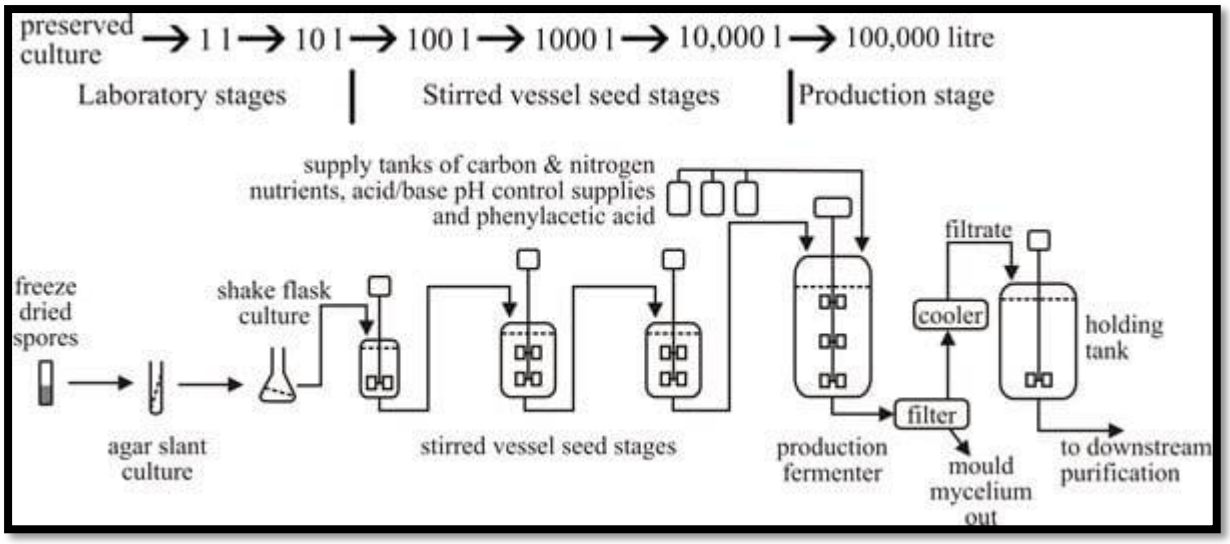
1. The catalytic step involves an ACV synthetase enzyme that condenses the lateral chain of cysteine, valine, and alpha aminoadipate into tripeptide ACV.

2. In the second step, tripeptide ACV forms a bicyclic ring by oxidative ring closure. Isopenicillin N synthase is involved resulting in isopenicillin N which is a bioactive intermediate in the pathway.
3. The third step involves the exchange of L-aminoadipate. Acyl-CoA synthetase and Acyl-CoA racemase, a two enzyme system is involved that helps in converting isopenicillin N into Penicillin N.



Penicillin Production Process

Penicillin production is done by fermentation process in a fermenter by agitating the culture of *P.chrysogenum* in a suitable condition. The whole process carried out is aerobic and the method involved is fed-batch.



This fermentation process is of Penicillin G which involves the following steps:

1. 100ml medium with spores of *P.chrysogenum* strains is inoculated in Erlenmeyer flask and is incubated at BOD incubator by placing them on a rotatory shaker.
2. After 4 days of incubation, the content along with two liters of medium is transferred into a flask that contains four liters and again incubates for two days.
3. Then, the content is transferred into a stainless tank containing 500 ml of the medium that provides suitable conditions for fungal growth.
4. After three days of incubation, the content is used for inoculation and kept in a fermentor that is well equipped with optimum conditions.
5. The content is filtered after six days of incubation which contains penicillin.
6. The penicillin is extracted into amyl or butyl acetate and is transferred into an aqueous solution with phosphate buffer.
7. Acidify the extract and again re-extract penicillin into butyl acetate
8. In the solvent extract potassium acetated is added to a crystallization tank to crystallize as a potassium salt.
9. Crystals were recovered and further sterilization of salt is done.

Application/Uses of Penicillin

- Used in treating infections caused by both Gram-positive and Gram-negative bacteria like respiratory tract infections, throat, mouth, gum, and urine infections and also used in treating bacterial endocarditis.

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Isolation of Microorganisms

➢ Microorganisms occur in natural environment like soil. They are mixed with several other forms of life. Many microbes are pathogenic. They cause a number of diseases with a variety of symptoms, depending on how they interact with the patient. The isolation and growth of suspected microbe in pure culture is essential for the identification and control the infectious agent.

➢ The primary culture from natural source will normally be a mixed culture containing microbes of different kinds. But in laboratory, the various species may be isolated from one another. A culture which contains just one species of microorganism is called a pure culture. The process of obtaining a pure culture by separating one species of microbe from a mixture of other species, is known as isolation of the organisms.

Methods of Isolation

➢ There are special techniques employed to obtain pure cultures of microorganisms. In few cases it is possible to secure pure culture by direct isolation or direct transfer. This can be done only in those situations in which pure culture occurs naturally. Kinds of specimens taken for culturing will depend on the nature and habitat of microbes.

➢ Different pathogens can be isolated from body tissues and fluids such as blood, urine, sputum, pus, faces, spinal fluid, bile, pleural fluids, stomach fluids etc. In the blood stream of a patient suffering with typhoid fever, the bacteria Salmonella typhii may be present. ➢ A pure culture of this bacterium may be obtained by drawing blood sample using a sterilized hypodermic syringe and treating the blood with anticoagulant such as heparin and potassium oxalate. The presence of the anticoagulant prevents the pathogenic microbe from entrapping in

fibrin clot. The sample of the blood may be inoculated into a suitable medium. Following isolation methods are employed to isolate microbes from mixed cultures

Streaking - This is most widely used method of isolation. The technique consists of pouring a suitable sterile medium into sterile petriplate and allowing the medium to solidify.

By means of a sterile loope or straight needle or a sterile bent glass-rod a small amount of growth preferably from a broth culture or bacterial suspension is streaked back and forth across the surface of agar until about one third of the diameter of the plate has been covered. The needle is then flamed and streaking in done at right angles to and across the first streak. This serves to drag bacteria out in a long line from the initial streak. When this streaking is completed the needle is again flamed and streaking is done at right angles to the second streak and parallel

2. Plating - It includes diluting of a mixture of microorganisms until only a few hundred bacteria are left in each milli litre of the suspension. A very small amount of the dilution is then placed in a sterile petri plate by means of a sterile loop or pipette. The melted agar medium is cooled to about 45°C and is poured into plate. The microorganism and agar are well mixed. When the agar is solidified the individual bacterium will be held in place and will grow to a visible colony.

3. Dilution - This method is used for the microorganisms which cannot be easily isolated by streaking or plating method. Sometimes when several organisms are present in a mixture, with one organism predominating, the predominating form may be isolated by this method. For example, when raw milk is allowed to sour at room temperature it will, at the time of curding, have a mixture of microorganisms with high percentage of Streptococcus. If 1 ml of the sour milk is taken into a tube containing 9 ml. of sterile milk (in which no organisms are present) then 1 ml. of this mixture is transferred with a sterile pipette into a second tube of sterile milk and the procedure is repeated i.e. from second to third tube, third to fourth tube until a series of about 10 tubes are inoculated.

Enrichment Procedure - This procedure involves the use of media and conditions of cultivation which favors the growth of the desired species.

For example, when a man suffers with typhoid, the intestinal discharge posses small number of Salmonella when compared with E. coli and other forms. It is almost impossible to isolate the typhoid organisms because they represent only a fraction of a per cent of the total microorganisms present. The media are therefore derived, which allow the rapid growth of the desired organisms, at the same time inhibiting the growth of other microorganisms.

5. Single Technique –

This is one of the most ideal and difficult method of securing pure culture. In this method a suspension of the pure culture is placed on the under-side of a sterile cover-glass mounted over a moist chamber on the stage of the microscope. While looking through the microscope, a single cell is removed with the help of sterile micropipette and transferred to a small drop of sterile medium on a sterile cover-glass and is mounted on a sterile hanging drop slide, which is then incubated at suitable temperature. If the single cell germinates in this drop, few cells are transferred into a tube containing sterile culture medium which is placed in the incubator to obtain pure culture originated from single cell

Cultivation of Microorganisms

➢ For cultivating microbes in laboratory, we require culture media. The various mixtures of nutritive substances used for the laboratory cultivation of microorganisms are collectively known as culture media. The culture media serve as soil in which bacteria are planted for the purpose of study.

Culture Media

➢ Culture media must contain all the essential nutrients required by the organism for its growth and reproduction. A suitable source of energy, building materials and growth factors must be supplied in adequate amounts. So a culture medium must contain:

➢ Since microorganisms show a considerable variation in their nutritional requirements, no single medium is suitable for growth of all of them.

➢ **The different types of culture media employed fall into three groups:**

1. Defined or synthetic media: These are the media prepared from chemical compounds. They are highly purified and specific that an investigator working in another laboratory can duplicate them.

2. Complex or non-synthetic media: Media that is prepared from ingredients that have not been precisely defined. It contains hydrolyzed proteins and vitamin extracts. This type of medium cannot be duplicated by another worker in another laboratory. Peptone is usually produced by boiling beef, by the hydrolysis of its protein. Casein peptone and milk peptone are also used in complex media as the source of amino acids and nitrogen. All liquid media, whether complex or synthetic may be converted to solid media by adding either gelatin (a protein) or agar-agar, (a complex polysaccharide) extracted from red marine algae.

The use of agar has an advantage. The most bacteria are unable to hydrolyze this molecule into more simple components. Since gelatin is a liquid at room temperature, the use agar allows the medium to remain in a solid form while microbes are growing on its surface.

3. Living cells: These are used for the cultivation of viruses. For example, fertilized eggs incubated for 8 to 12 days are able to support the growth of many viruses.

➢ In another classification culture media are grouped into following four types:

1. Natural media: These include substances occurring in nature such as milk, eggs, blood, extract of plant and animal tissues.

2. Derived media: These include known substances but the chemical composition of each is unknown.

Examples are

1. Nutrient broth (prepared by boiling beef to extract nutrients and adding an amino acid-nitrogen source.)
2. Nutrient agar
3. Nutrient gelatin.

3. Chemically defined media: Exact chemical composition of these media is known.

4. Special media: These include combinations of the other three types of media.

➢ Categories of media used in laboratory:

1. **Enrichment media:** They are prepared with ingredients that will enhance the growth of certain microbes. Enrichment media encourage the growth of the suspected pathogen so that it will become the most pre-dominant type of microbe in the culture. Two types of enrichment media are blood agar and chocolate agar.

2. Selective media: They are prepared with ingredients that inhibit the growth of unwanted microbes which might be in the specimen. The inhibitor may be an antibiotic, salt or other chemical. Mixed culture of microbes originally grown in enrichment media may be inoculated into selective media to isolate the desired microbe

3. Differential media: They are designed to differentiate among microbes. Different bacterial species may produce dissimilar colony colours when grown on differential agar. While in differential broth cultures, the media change colour. Differential media are used to confirm the identity of a microbe that has already been isolated by culturing in enrichment and selective media.

4. Propagation media: They are used to propagate, or keep microbes growing for a long lime. Samples grown on these media may be taken for analysis. The most common propagation media are nutrient broth and agar.

Preparation of Media

➢ There are three main steps in the preparation of media: Preparation as solutions of chemicals and adjusting the pH, dispensing the media, and sterilization.

➢ A broth is prepared by dissolving the appropriate amount of the components in distilled water and pH is adjusted by the addition of either dilute NaOH or HCl. The broth is dispensed into clean rimless 'Pyrex' test tubes which are plugged with non-absorbent cotton wool plugs. The test tubes are placed in wire baskets which are covered with grease proof paper.

➢ The media are sterilized by autoclaving at a temperature of 121°C and a pressure of 151 b/in2 for 15 minutes. But medium containing heat- sensitive substances are sterilized either by filtering the solution at room temperature, using bacteria-proof filter or by a process called Tyndallization.

➢ In this method, the liquids are steamed for one hour a day on three consecutive days and the liquids are incubated at 25-30°C. During the first steaming, all the heat sensitive vegetative cells are killed, leaving only the spores. During the first incubation period, most of the spores germinate in to vegetative cells. These vegetative cells are killed by the second steam period.

➢ In the second incubation period, the rest of the spores germinate into vegetative cells which are killed by the third steaming period. In this way, the liquids are sterilized without temperature rising above 100°C.

Maintenance of Pure Culture

➢ After obtaining the pure culture of a particular microbe, it may be grown and maintained as a pure culture in different ways:

➢ The most common practice is to grow the culture on suitable medium until it reaches the stationary phase of growth, and then store in a refrigerator. If they are to be kept alive for a long period all culture must be transferred to a fresh sterile medium. Thus by successive transfer, a culture may be kept for an indefinite period.

➢ A second method involves freezing of young culture and desiccating it under vacuum. The cells of a pure culture will remain viable for a long period of time if they are mixed with sterile blood serum, sterile skimmed milk, before freezing and drying. They dried cultures are kept in the sealed, evacuated tubes and are stored in cool places.

➢ This method of maintaining pure culture is most suitable for spore forming species. The microorganisms are grown in pure culture in suitable media. A suspension of microorganisms is then transferred to cotton stoppered tubes of sterilized dry soil. The spores remain viable, though dormant, for long periods of time, in dry soil. The organism can be grown after a desired

period, by transferring the soil into a suitable medium and incubating it under suitable temperature.

Improvement of Industrially Important Microorganisms

Strain improvement of microorganisms is an important tool for improving the productivity of bioprocesses. In general, there are three main strategies for improving the productivity of a microorganism:

1) Selection of high-yielding mutants: This approach involves screening a population of microorganisms for variants that produce more product than the parental strain. The most common method for selecting high-yielding mutants is to plate cultures on media containing a selection agent that is toxic to the parental strain but not to the mutant. High-yielding mutants can then be isolated by growing colonies from the plates in liquid culture and selecting for growth on minimal media.

2) Genetic engineering: This approach involves manipulating the genetic sequence of a microorganism to improve its productivity. One common strategy is to insert genes from other organisms that encode enzymes that catalyze the production of the desired product.

3) Metabolic engineering: This approach involves altering the metabolic pathways of a microorganism to redirect its resources towards the production of the desired product. One common strategy is to knock out genes that are not essential for product formation and replace them with genes from other organisms that encode enzymes that catalyze the production of the desired product.

Media Formulation

• Formulation of medium is an vital phase in pilot-scale expansion, laboratory experiments and developmental processes.

• Few are some measures important to consider while manipulating a medium for the purpose of huge range of production:

- It should produce maximum product.
- It should give minimum yield of undesired product.
- It should be cheap.
- It should cause minimal problems in aeration, medium formation, sterilization, agitation, extraction, purification and waste treatment.
- It should produce maximum concentration of biomass and must be available throughout the year.
- Media must satisfy all nutritional requirements of the organism and fulfil the objectives of the process

Components of Media

• Fermentation medium consists of macronutrients, micronutrients, trace elements, dissolved oxygen, vitamins, enzymes, other dissolved gases, and inhibitors.

• The components of the medium should accomplish the elemental necessities for metabolite construction and biomass production with sufficient provision of energy for biosynthesis (Springham & Moses, 1999).

Carbon + Nitrogen + Oxygen + Other requirements -- -----› Biomass + Product + Carbon dioxide + Water + Heat Water and Energy Sources

• Chief constituent of every fermentation medium is water and it is required in rinsing, cooling, and heating.

• It is significant to consider the dissolved salts, contamination, effluents, and pH, while evaluating the water supply.

• Water's mineral contents play an important function in brewing and are critical in squashing.

• Light or medium components of oxidation are main source of energy which is required for growth.

• As industrial microbes are chemoorganotrophs, so their source of energy is carbon source in form of lipids, proteins, and carbohydrates.

• While, in some cases, methanol or hydrocarbons may be used by some microorganisms as a source of energy or carbon

Carbon Source

• Carbon is considered as a main product of a fermentation process.

• If 60-70% of production cost is raw materials during single-cell protein or ethanol production, then the product's selling price will be indicated by expense of the carbon supply. Carbohydrates

• Starch obtained from cereals, potatoes, and maize, is easily available as a source of carbohydrates and are extensively used in fermentation of alcohol.

• Grains (maize etc) are used in the form of powder and also as a source of carbohydrates

• Cheapest source of carbohydrates is molasses, and used in organic acid, amino acid, single-cell protein, and alcohol fermentations

Fats and Oils

• Oils were firstly used as antifoaming agents in antibiotic processes

• Oils provide maximum energy per weight than sugars.

• Oils posses anti-foaming qualities but are used as additives.

• These may also be used for their high content of the fatty acids.

Nitrogen Source

• Industrially used microorganisms have ability to use organic as well as inorganic means of nitrogen.

• Inorganic source of nitrogen is supplied as ammonium salts, ammonia gas and nitrates

• Inorganic substrates which can be used as a source of nitrogen includes urea, ammonium salts, and ammonia.

• Ammonia is used to control pH during fermentation process.

Minerals

• Essential minerals which are used in all media formulation include potassium, sulphur, chlorine, phosphorous, magnesium, and calcium

• We require a minute amounts other minerals such as cobalt, manganese, zinc, iron, copper and molybdenum and they exist as impurities.

• The specific concentration of these all elements depends on the micro-organism.

Chelators

• Metal precipitation is avoided by addition of chelating agents.

• In large scale fermentation chelating agents are not necessary.

• Some other ingredients (yeast extract) will play role of formation of metal ion complexes.

• As EDTA is capable of forming bonds with magnesium and calcium ions thus they are widely used in soaps and detergents.

Growth Factors

• Few of the microbes cannot produce complete complement of components of the cell and consequently requisite some of the preformed components known as growth factors.

• Growth factors includes amino acids, vitamins, sterols and fatty acids.

• Some natural sources such as nitrogen and carbon are used in growth medium formulations having required growth factors.

• Cautious mixing of materials can be used to eliminate the vitamin deficiency.

Buffers

• pH has great influence on microbial growth.

• pH of the growth media can be maintained by addition of buffers that would resist pH changes.

• Many microorganisms have optimum pH range 7.0.

• Some of the examples of buffers that are commonly used include; ammonia sodium hydroxide and calcium carbonate.

Media Sterilization

• In industrial fermentations, components such as vessels, pipework, media, inlet air, and exhaust gases are frequently sterilized by a combination of wet-heat and filtration methods.

• Wet-heat methods are less expensive and more effective than dry-heat methods, and thus are employed commonly in fermentation industries to destroy unwanted microorganisms.

• The wet-heat sterilization conditions typically used to kill all microorganisms, including bacterial spores, are listed in Table 1. Physical Methods

• The physical methods such as filtration, centrifugation, and adsorption (to ion-exchangers or activated carbon) are in use.

• Among these, filtration is most widely used.

• Certain constituents (vitamins, blood components, antibiotics) of culture media are heat labile and therefore, are destroyed by heat sterilization.

• Such components of the medium are completely dissolved and then subjected to filter sterilization.

• There are a couple of limitations of filtration technique:

– Application of high pressure in filtration is unsuitable for industries.

– Some of the media components may be lost form the media during filtration.

– Sometimes, a combination of filtration and heat sterilization are applied.

– For instance, the water used for media preparation is filtered while concentrated nutrient solution is subjected to heat sterilization.

– The filtered water is now added for appropriate dilution of the media. • The chemical methods (by using disinfectants) and radiation procedures (by using UV rays, y rays, X-rays) are not commonly used for media sterilization.

Batch Sterilization

• The culture media are subjected to sterilization at 121°C in batch volumes, in the bioreactor.

• Batch sterilization can be done by injecting the steam into the medium (direct method) or injecting the steam into interior coils (indirect method).

• For the direct batch sterilization, the steam should be pure, and free from all chemical additives (that usually come from steam manufacturing process). There are two disadvantages of batch sterilization:

1. Damage to culture media:

• Alteration in nutrients, change in pH and discolouration of the culture media are common.

2. High energy consumption:

• It takes a few hours (2-4 hrs.) for the entire contents of the bioreactor to attain the requisite temperature (i.e. 120°C).

• Another 20-60 mins for the actual process of sterilization, followed by cooling for 1-2 h

• This process involves wastage of energy, and therefore batch sterilization is quite costly.

Continuous Sterilization

• Continuous sterilization is carried out at 140°C for a very short period of time ranging from 30 to 120 seconds.

• Continuous sterilization is carried out by directly injecting the steam or by means of heat exchangers.

• In either case, the temperature is very quickly raised to 140°C, and maintained for 30- 120 seconds.

• The different stages are— exchanger, heater, heat maintenance unit, recovery of residual heat, cooling and fermenter.

• In the continuous sterilization process, 3 types of heat exchangers are used.

– The first heat exchanger raises temperature to 90-120°C within 20-30 seconds.

– The second exchanger further raises temperature to 140°C and maintains for 30-120 seconds.

– The third heat exchanger brings down the temperature by cooling in the next 20-30 seconds.

• The actual time required for sterilization depends on the size of the suspended particles. The bigger is the size, the more is the time required.

• The main advantage with continuous sterilization is that about 80-90% of the energy is conserved.

• The limitation however, is that certain compounds in the medium precipitate (e.g., calcium phosphate, calcium oxalate) due to very high temperature differences that occur in a very short time between sterilization and cooling.

• The starch-containing culture media becomes viscous in continuous sterilization and therefore is not used.