PROCESSES OF RECOMBINANT DNA TECHNOLOGY

The primary steps involved in recombinant DNA (rDNA) technology are outlined as follows:

1) Extraction of Genetic Material (DNA): The initial step involves isolating the genetic material, specifically DNA, from the source organism.
2) DNA Fragmentation: The DNA is then fragmented at precise locations using restriction endonucleases, enzymes that cleave DNA at specific sequences.
3) Separation and Isolation of DNA Fragments: Following fragmentation, the DNA fragments are separated and isolated based on their size using techniques such as gel electrophoresis.
4) Gene Amplification via PCR: Polymerase Chain Reaction (PCR) is employed to amplify specific DNA segments, enabling the production of numerous copies of the desired gene.
5) Insertion of Recombinant DNA into Host Cells/Organisms: The amplified DNA fragments, now known as recombinant DNA, are inserted into the host cells or organisms using various methods such as transformation, transfection, or viral transduction.
6) Cultivation of Host Cells/Organisms: The host cells containing the recombinant DNA are cultured on a large scale in a nutrient-rich medium to facilitate the expression of the foreign gene product.
7) Extraction of Desired Product Utilizing Downstream Processing: Finally, the desired product, whether it be a protein, enzyme, or other biotechnological product, is extracted and purified through downstream processing techniques for further use or analysis.

1. DNA Isolation

The process of isolating DNA involves extracting both the passenger DNA and the vehicle DNA from their respective cellular environments. To achieve this, cells are lysed using appropriate enzymes, thereby breaking open the cell membranes to release DNA along with various other macromolecules such as RNA, proteins, polysaccharides, and lipids.

Different enzymes are utilized based on the source of DNA.

For instance, lysozyme is employed for bacterial cells, cellulase for plant cells, and chitinase for fungi. It's important to note that genes are situated on long DNA molecules intertwined with proteins like histones.

To further purify the DNA, RNA can be eliminated through treatment with ribonuclease, while proteins can be removed by treating with protease. Additional molecules can be eliminated through appropriate treatments. Ultimately, purified DNA precipitates out upon the addition of chilled ethanol, appearing as fine threads within the suspension. The separated DNA can then be removed using the spooling method.

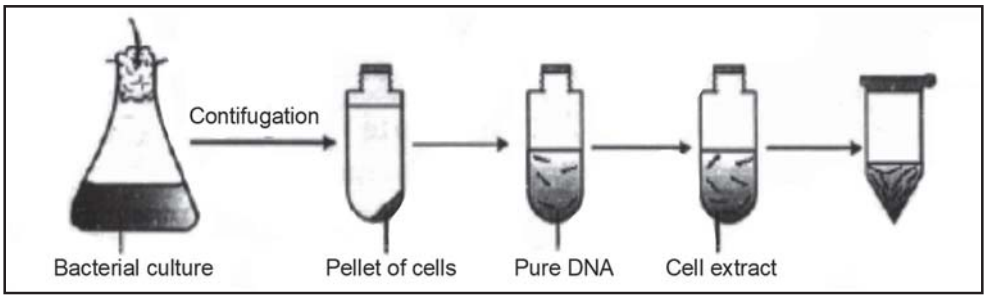


Fig.: The fundamental stages involved in the extraction of total cellular DNA from a bacterial culture.

The process of extracting total DNA from a bacterial cell culture involves several detailed steps, as depicted in the figure above:

1) Cultivation and Harvesting: Initially, a bacterial culture is cultivated and allowed to grow to an optimal density. Subsequently, the culture is harvested at a specific growth stage.

2) Cell Disruption: The harvested bacterial cells undergo a process of disruption to release their contents. The method of disruption varies depending on the type of cell:
   - For bacterial cells, the enzyme lysozyme is utilized.
   - Fungal cells are disrupted using chitinase.
   - Plant cells are disrupted using cellulase.

3) Extraction of DNA: Following cell disruption, the resulting cell extract undergoes treatment to eliminate all components except for the DNA. This process involves the removal of RNA molecules by treatment with ribonuclease and the removal of proteins by treatment with protease.
   During this process, it is important to note that genes are situated on elongated DNA molecules intertwined with proteins like histones. Ribonuclease treatment specifically targets and eliminates RNA molecules, whereas protease treatment targets and eliminates proteins, facilitating the isolation of pure DNA.

4. The DNA solution obtained undergoes a concentration process. Any remaining molecules other than DNA can be eliminated through suitable treatments. Eventually, the purified DNA precipitates out when chilled ethanol is added. This precipitation can be observed as the formation of fine threads within the suspension, as illustrated in the figure below.

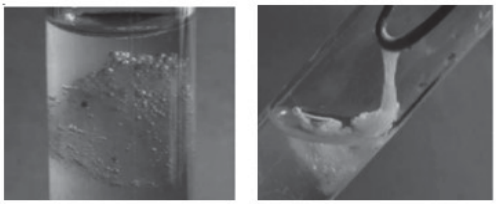


Fig. : DNA that separates out can be removed by spooling

Example: Identify the reagent responsible for causing DNA precipitation from a solution.
Solution: Chilled ethanol.

Example: Within an E. coli cell, DNA is confined within membranes. Propose a reagent or molecule capable of liberating entrapped DNA.
Solution: Lysozyme.

(2) Cutting of DNA at Specific Locations

To achieve fragmentation of DNA at specific locations, restriction enzyme digestions are conducted. This involves incubating purified DNA molecules with the restriction enzyme at a temperature of 37°C in a buffer solution to maintain the pH and provide necessary water content. The progress of the restriction enzyme digestion is monitored using agarose gel electrophoresis. It's essential that both the vector and the insert are digested using compatible restriction enzymes, meaning they should be the same enzyme for efficient digestion.

(3) Separation and Extraction of DNA Fragments

A. Gel Electrophoresis for DNA Fragment Separation
   - When DNA is cleaved by restriction endonucleases, it yields various fragments of DNA. These fragments can be isolated using a technique called gel electrophoresis.
   - Since DNA fragments are negatively charged molecules, they can be separated by applying an electric field that forces them to migrate towards the positive electrode (anode) through a medium or matrix.

- Currently, the most commonly used matrix for this purpose is agarose, a natural polymer extracted from seaweeds. The DNA fragments undergo separation based on their size, facilitated by the sieving effect created by the agarose gel. Consequently, smaller DNA fragments migrate farther distances during the electrophoresis process.

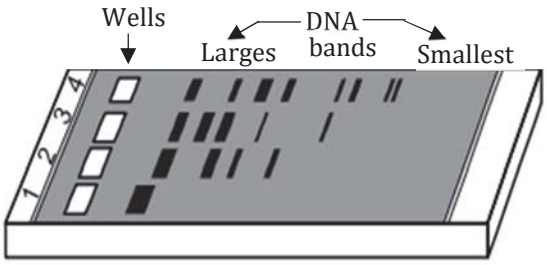


Figure: Illustration of a Standard Agarose Gel Electrophoresis Displaying the Migration

- Agarose gel electrophoresis is utilized to monitor the progress of restriction enzyme digestion, a process repeated with the vector DNA as well.
- The separated DNA fragments are only visible after staining with a compound called ethidium bromide, an intercalating dye, followed by exposure to UV radiation. Pure DNA fragments are not visible under visible light without staining. When viewed under UV light, the DNA fragments appear as bright orange-colored bands within the ethidium bromide-stained gel.

B. Extraction of Targeted DNA Fragment

The bands of separated DNA are excised from the agarose gel and isolated from the gel fragment. This process is termed as elution. The DNA fragments purified through this method are employed in the construction of recombinant DNA by incorporating them into cloning vectors.

(4) Amplification of Gene of Interest using PCR

PCR, an abbreviation for Polymerase Chain Reaction, is a groundbreaking technique that has revolutionized the field of biotechnology. Similar to the significance of the light microscope introduced a century ago, the PCR machine has recently become an indispensable tool in biological research. Despite its recent prominence, the concept of PCR emerged only in the early 1970s. However, it took more than a decade before American biochemist Kary Mullis translated this idea into reality.

PCR enables the rapid production, or amplification, of billions of copies of a specific segment of DNA within minutes or hours, contrasting sharply with traditional methods that could take days.

Essentially, PCR operates as DNA replication on a larger scale. The polymerase chain reaction relies on several essential chemical components:

- A DNA polymerase: In the early stages of PCR, a significant limitation was the necessity to add fresh DNA polymerase during each cycle. Mullis and his colleagues addressed this issue by demonstrating how a particular type of thermostable DNA polymerase, known popularly as "Taq polymerase," derived from the heat-resistant bacterium Thermus aquaticus, eliminated the need for fresh polymerase addition throughout the cycle. Thermus aquaticus, a thermophilic bacterium thriving in extreme temperatures up to 95°C, naturally inhabits the hot spring ecosystem of Yellowstone National Park. This innovation greatly enhanced the quantity and quality of PCR products.
- A small amount of DNA to serve as the initial template (nanograms).
- The four types of deoxyribonucleotides, acting as substrates for the DNA polymerase and providing the raw materials for the formation of new DNA molecules.
- Several necessary ions and salts.

- A pair of primers, small chemically synthesized oligonucleotides with exposed 3'-OH groups, bind to specific regions of the DNA template.

These primers are essential for initiating DNA replication. DNA polymerases can only add new nucleotides to the 3'-OH end of a growing strand, necessitating the presence of a primer to commence synthesis. Two primers are required - one for each of the two DNA strands - known as the forward primer and reverse primer.

A single PCR reaction involves three temperature-dependent steps:

(i) Denaturation: The initial solution is heated, usually to 94°C. High temperatures break the hydrogen bonds between the two strands of the original DNA double helix, providing the necessary single-stranded templates.

(ii) Annealing: After heating, the reaction mixture is rapidly cooled, typically to a temperature between 50° and 60°C, where it is held for less than a minute. During this time, the primers bind to their complementary sequences on the single-stranded templates.

(iii) Primer extension (polymerization): The sample is then heated to 72°C for a certain duration, during which the DNA polymerase adds nucleotides to the primer, synthesizing a new DNA strand using only the template sequences that bind the primers.

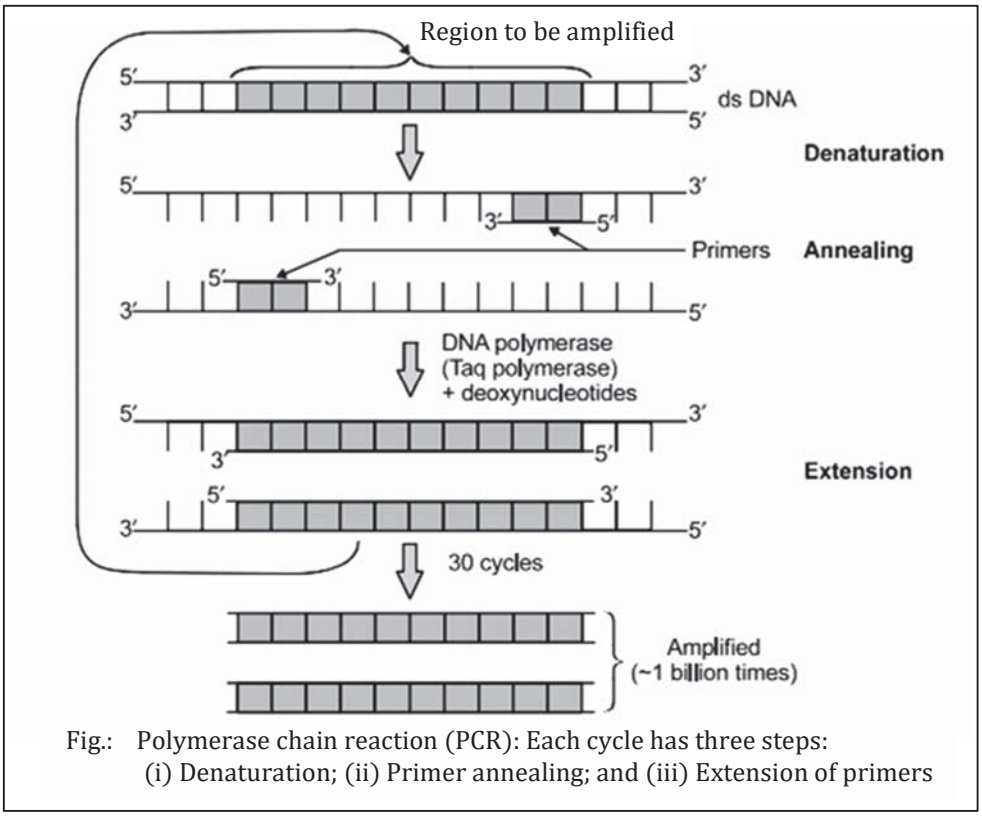


Fig.: Polymerase chain reaction (PCR): Each cycle has three steps:
(i) Denaturation; (ii) Primer annealing; and (iii) Extension of primers

If the DNA replication process is iterated numerous times, the DNA segment can be amplified to approximately a billion times, resulting in the production of one billion copies by the conclusion of 30 PCR cycles. After 'n' number of cycles, it is feasible to generate 2^n molecules.

Applications of PCR:

(i) Pathogen Diagnosis: Pathologists utilize techniques centered on identifying specific enzymes or antibodies against disease-related proteins. However, these methods encounter limitations in detecting infectious agents that are challenging to culture or persist at very low levels in infected

cells. To address these challenges, PCR-based assays have been devised to detect the presence of gene sequences of infectious agents.

(ii) Detection of Specific Mutations: PCR can be employed to identify the presence of particular mutations responsible for causing specific genetic diseases before the actual manifestation of the disease. By utilizing PCR, diseases such as phenylketonuria, muscular dystrophy, sickle cell anemia, AIDS, hepatitis, chlamydia, and tuberculosis can be diagnosed.

(5) Integration of the DNA Fragment into Vectors

Once the DNA fragment has been amplified, if necessary, it can be prepared for integration into a vector for subsequent cloning purposes.

(6) Integration of Recombinant DNA into Host Cells/Organisms

Various methods exist for introducing the ligated DNA into recipient cells. The recipient cells, once rendered 'competent' to receive the DNA, uptake the DNA present in their surroundings.

As DNA is a hydrophilic molecule, it cannot traverse cell membranes spontaneously. Therefore, to compel bacteria to accept the plasmid, the bacterial cells must first be made 'competent' to uptake DNA. This is achieved by subjecting them to a specific concentration of a divalent cation, such as calcium, which enhances the efficiency of DNA entry into the bacterium through pores in its cell wall. Calcium chloride is presumed to either cause DNA to precipitate onto the outer surface of the cells or enhance DNA binding.

(i) Transformation: Recombinant DNA can be introduced into these cells by incubating them with the recombinant DNA while on ice. Subsequently, the cells are briefly subjected to a heat shock at 42°C before being returned to ice. This process facilitates the uptake of recombinant DNA by the bacteria.

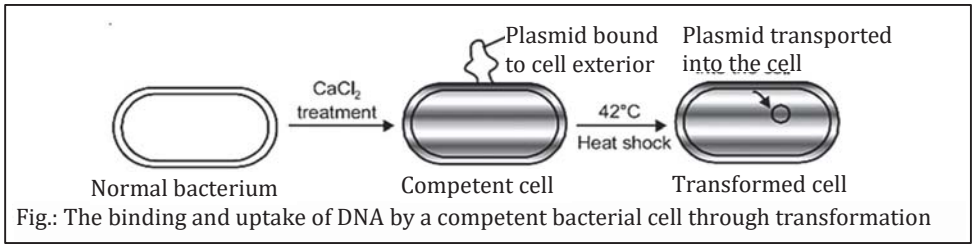


Fig.: The binding and uptake of DNA by a competent bacterial cell through transformation

This process is known as transformation, which is a method used to introduce a specific DNA fragment into a host bacterium.

For instance, if a recombinant DNA containing a gene for resistance to an antibiotic, such as ampicillin, is transferred into E. coli cells, the host cells undergo transformation and become resistant to ampicillin. When the transformed cells are plated on agar plates containing ampicillin, only the transformed cells survive and grow, while the untransformed recipient cells perish. This ability to selectively grow transformed cells in the presence of ampicillin highlights the significance of the ampicillin-resistance gene as a selectable marker.

(ii) Another technique, termed microinjection, involves the direct injection of recombinant DNA into the nucleus of an animal cell.

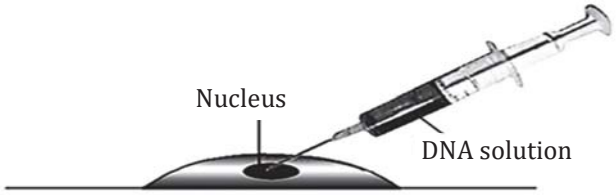


Fig.: Microinjection

(iii) Another method, applicable to plants, involves bombarding cells with high-velocity microparticles made of gold or tungsten that are coated with DNA. This technique is known as biolistic or gene gun.

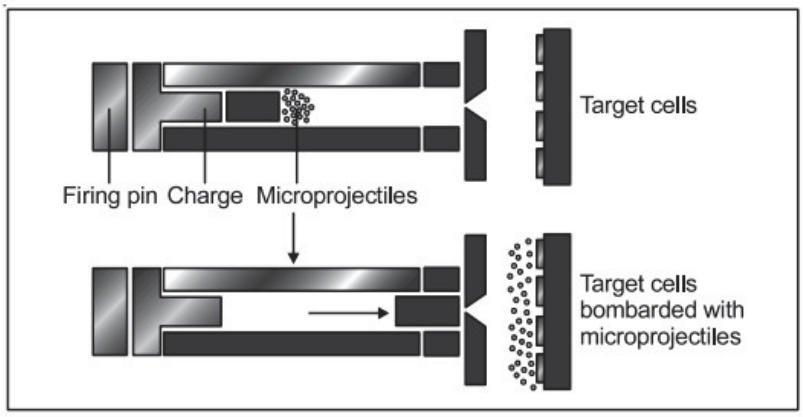


Fig. : Transformation with microprojectiles

(iv) Utilizing 'disarmed pathogen' vectors, this method involves the transfer of recombinant DNA into the host cell when the vectors infect the cell. Examples of such vectors include disarmed Ti plasmids from Agrobacterium and disarmed Retroviruses.

(v) Electroporation: This technique involves applying short electrical impulses of high field strength, which serve to enhance the permeability of the protoplast membrane. This process creates transient microscopic pores, thereby facilitating the entry of DNA molecules into the cells more efficiently.

(7) Scaling Up Host Cell Cultures in a Nutrient Medium for Large-Scale Production of Foreign Gene Products/ Obtaining the Foreign Gene Product

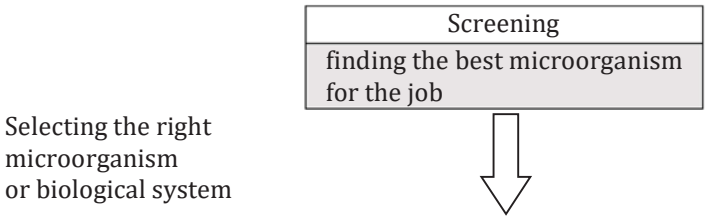
The primary objective of Recombinant DNA Technology (RDT) is to generate significant quantities of a desired protein. To achieve this, protein expression is crucial, and inducers are often employed to boost the production of the targeted protein or gene of interest.

When a protein-encoding gene is expressed in a host organism different from its native one, it is termed as a recombinant protein.

For instance, the expression of the human insulin gene in a vector within E. coli serves as a suitable example where bacteria act as a heterologous host.

Transitioning from laboratory-scale to industrial-scale production poses new challenges for biotechnologists. Before implementing any new biotechnological manufacturing processes, they must be thoroughly tested at a laboratory scale. The cells containing the cloned genes of interest may be cultured on a small scale in the laboratory. Subsequently, the culture can be utilized to extract the desired protein, which is then purified using various separation techniques.

The accompanying figure or flow diagram illustrates some key stages typically involved in establishing a biotechnological process.



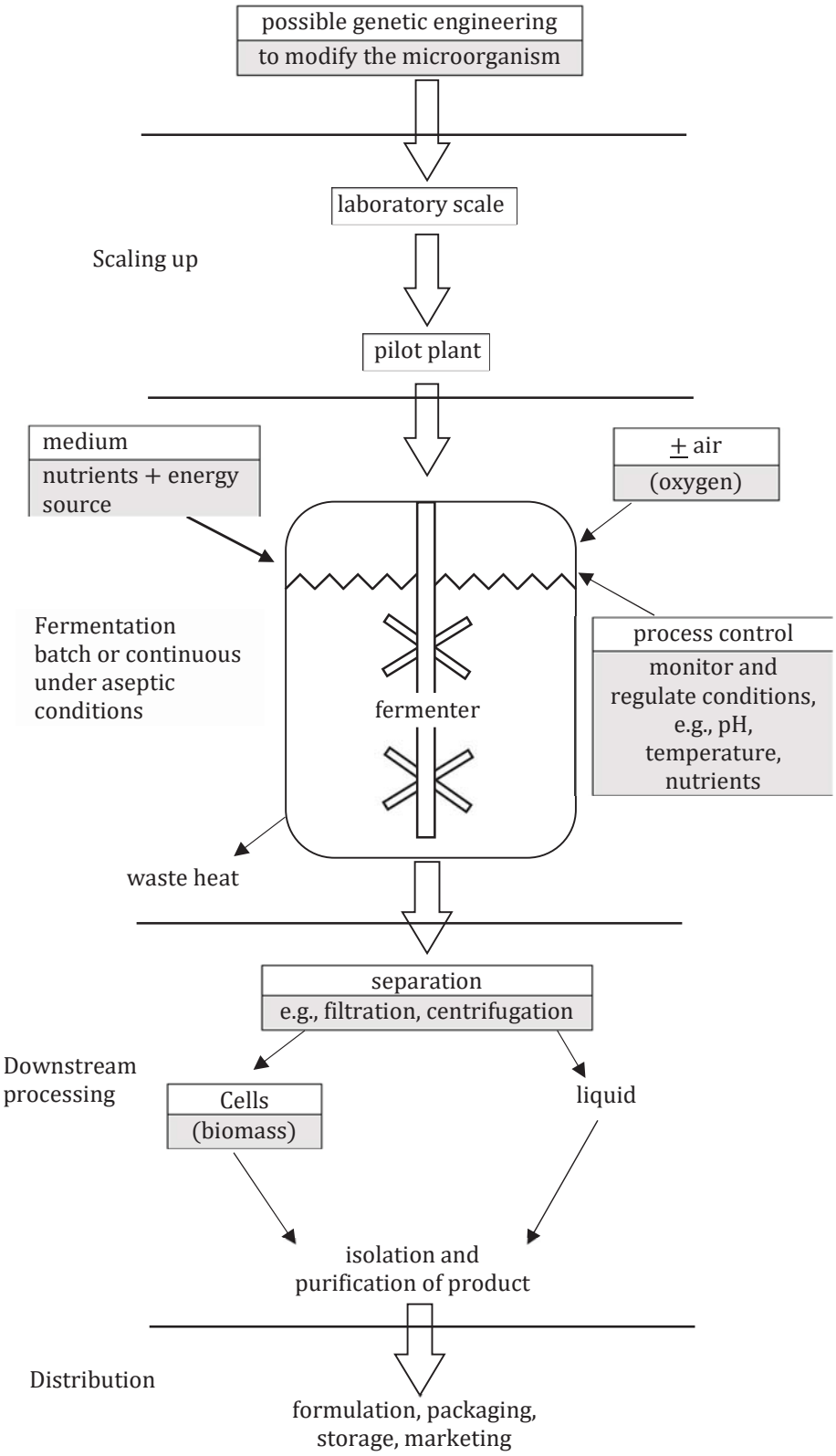


Figure: Outline of a biotechnological procedure

Following initial investigations conducted using standard laboratory equipment, it is customary to establish a 'pilot plant', which involves utilizing a small-scale 'fermenter', such as large shake flasks found in laboratories. A fermenter serves as the vessel or tank where the biotechnological process will

be executed. It is imperative to ascertain the optimal nutrient and physical conditions to achieve maximum yield.

Numerous new factors come into consideration when transitioning the process from pilot production to full-scale (100-1000). Some of the crucial factors include:

- Maintaining Aseptic Conditions: Preventing contamination of both inputs and outputs to the main fermenter is essential.
- Addressing Physical Factors: Challenges such as mixing, aeration of the media, and managing waste heat pose significant obstacles when scaling up the process.
- Oxygen Supply in Large-Scale Cultures: In large-scale cultures, it becomes necessary to force air through the medium to ensure sufficient oxygen supply. Simple agitation methods used at the laboratory scale are insufficient. Spargers, tubes with small holes, are employed to generate small bubbles, which are more effective. Additionally, stirring may also be utilized.
- Use of Anti-Foaming Agents: Foaming caused by stirring and aeration requires the use of anti-foaming agents to reduce its impact.
- Managing Heat Generation: Microbial activity and large-scale production generate heat, necessitating the circulation of cooling water around the fermenter.
- Ensuring Consistent Conditions: Maintaining constant conditions such as nutrient supply, pH, and oxygen concentration throughout the medium on a large scale is crucial. This requires the implementation of sophisticated monitoring devices and control processes.

Fermenters, also referred to as bioreactors, serve as chambers where microorganisms are cultivated in either liquid or solid mediums. In order to produce large quantities of the desired product, bioreactors were developed to accommodate large volumes of culture, ranging from 100 to 1000 liters. Bioreactors function as vessels wherein raw materials undergo biological conversion into specific products, individual enzymes, etc., utilizing microbial, plant, animal, or human cells. These bioreactors provide the optimal conditions necessary for achieving the desired product by maintaining ideal growth conditions, including temperature, pH, substrate, salts, vitamins, and oxygen levels.

Design and Usage of Fermenters: The most commonly employed bioreactors are of the stirring type, as depicted in the figure provided below.

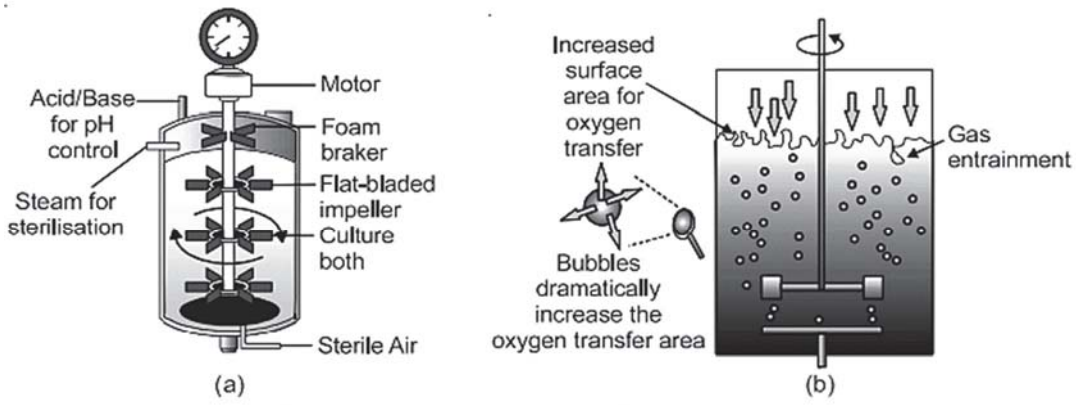


Fig. : (a) Simple stirred-tank bioreactor; (b) Sparged stirred-tank bioreactor through which sterile air bubbles are sparged.

Typically, a stirred-tank reactor adopts a cylindrical shape or features a curved base to enhance the mixing of its contents. The stirrer ensures uniform mixing and enables adequate oxygen distribution throughout the bioreactor. Alternatively, air can be introduced into the reactor through bubbling. A bioreactor system encompasses an agitator system, an oxygen delivery system, and a foam control system. It also includes temperature and pH control systems, along with sampling ports for periodic withdrawal of small volumes of the culture.

Types of Fermentation:
(i) Batch Fermentation (Closed System)
(ii) Continuous Culture (Open System)
In batch fermentation, the conditions are established and remain unchanged from the outside once fermentation commences.
For instance, no additional nutrients are introduced. This characteristic defines the process as a closed system. The fermentation process halts once an adequate quantity of product has been generated. Subsequently, the contents of the fermenter are extracted, the product is isolated, the microorganisms are discarded, and the fermenter is cleaned and prepared for a new batch.
In a continuous culture system, the utilized medium is drained out from one side, while fresh medium is added from the other to sustain the cells in their physiologically most active logarithmic/exponential phase. This culture method generates a larger biomass, resulting in higher yields of the desired protein. Shake flasks, which accommodate small volume cultures, are incapable of yielding appreciable quantities of products.
Continuous culture entails ongoing, long-term operation spanning several weeks, during which nutrient medium is replenished at the same rate it is utilized, and the surplus is harvested.

(8) Utilization of Downstream Processing for Extraction of the Desired Product

Downstream Processing

Downstream processing denotes the phase subsequent to fermentation, wherein the desired product undergoes recovery and purification. Upon completion of the biosynthetic phase, the product must undergo a sequence of procedures before it becomes market-ready as a finished product. These downstream processes encompass the separation and purification of the desired product.
Typically, the contents of the fermenter are initially segregated into a liquid component and a solid component containing cells. This separation is commonly achieved through filtration or centrifugation. The liquid component may contain the desired product in solution, or it may comprise cells or a product encapsulated within the cells.
A plethora of biochemical separation and purification techniques are available, including drying, chromatography, solvent extraction, and distillation.
Following purification, the product necessitates formulation with suitable preservatives. Such formulation must undergo rigorous clinical trials, akin to drugs. Additionally, stringent quality-control testing is imperative for each product. The downstream processing and quality-control procedures vary depending on the specific product.

Example: How are bacterial cells rendered competent?
Solution: Bacterial cells are made competent by subjecting them to a specific concentration of certain divalent cations.

Example: What is the transformation of bacterial cells?
Solution: Transformation of bacterial cells refers to the uptake of DNA by the bacterial cells.