CHAPTER – 11

BIOTECHNOLOGY: PRINCIPLES AND PROCESSES

Introduction

- Biotechnology: deals with techniques of using live organisms or enzymes from organisms to produce products and processes useful to humans.
- According to EFB: 'The integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services'.

Principles of biotechnology:

Genetic engineering: Techniques to alter the chemistry of genetic material (DNA and RNA), to introduce these into host organisms and thus change the phenotype of the host organism.

Bio process engineering: Maintenance of sterile (microbial contamination-free) ambience in chemical engineering processes to enable growth of only the desired microbe/eukaryotic cell inlarge quantities for the manufacture of biotechnological productslike antibiotics, vaccines, enzymes, etc.

Contribution of Stanley Cohen and Herbert Boyer

- Stanley Cohen andHerbert Boyer accomplished in 1972 constructed the first recombinant DNA by linking a gene encoding antibiotic resistance with a native plasmid (autonomously replicating circular extra-chromosomal DNA) of *Salmonella typhimurium*.
- It was accomplished by isolating the antibioticresistance gene by cutting out a piece of DNA from a plasmid which wasresponsible for conferring antibiotic resistance.
- The cutting of DNA atspecific locations became possible with the discovery of the 'molecular scissors'– restriction enzymes.
- The cut piece of DNA was then linked with the plasmid DNA. These plasmid DNA act as vectors to transfer the piece of DNA attached to it.

Tools of Recombinant DNA Technology

- The key tools of rDNA technology are: Restriction enzymes, polymerase enzymes, ligases, vectorsand the host organism.

Discovery of Restriction enzymes

- In the year 1963, the two enzymes responsible for restricting the growthof bacteriophage in *Escherichia coli* were isolated.
- One of these addedmethyl groups to DNA, while the other cut DNA. The later was calledrestriction endonuclease.

The first restriction endonuclease

- The first restriction endonuclease–Hind II, whose functioningdepended on a specific DNA nucleotide sequence was isolated andcharacterized.
- Later it was found that Hind II always cut DNAmolecules at a particular point by recognising a specific sequence ofsix base pairs.
- This specific base sequence is known as therecognition sequence for Hind II.

Types of restriction enzymes,

- Restriction enzymes belong to a larger class of enzymes callednucleases.
- These are of two kinds: exonucleases and endonucleases.
- Exonucleases remove nucleotides from the ends of the DNA whereas,endonucleases make cuts at specific positions within the DNA.Each restriction endonuclease functions by 'inspecting' the length ofa DNA sequence.
- Once it finds its specific recognition sequence, it will bind to the DNA and cut each of the two strands of the double helix at specific points in their sugar -phosphate backbones .
- Each restriction endonuclease recognises a specific palindromic nucleotide sequences in the DNA.
- This leaves single stranded portions at the ends. There are overhanging stretches called sticky ends on each strand.
- The sticky ends: These are named so because they form hydrogen bonds with their complementary cut counterparts. This stickiness of the ends facilitates the action of the enzyme DNA ligase.
- Restriction endonucleases are used in genetic engineering to form 'recombinant' molecules of DNA, which are composed of DNA from different sources/genomes.
- When cut by the same restriction enzyme, the resultant DNA fragments have the same kind of 'sticky-ends' and, these can be joined together (end-to-end) using DNA ligases.

Convention of naming restriction enzymes

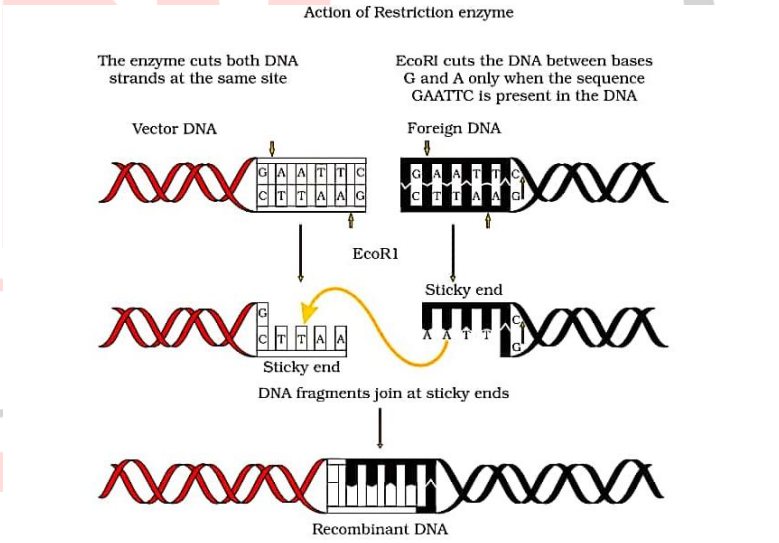
- The convention for naming restriction enzymes is the first letter of the namecomes from the genus and the second two letters come from the species ofthe prokaryotic cell from which they were isolated.
- For example EcoRI comes from*Escherichia coli* RY 13. 'E' from the genus *Escherichia* andnest two letters 'co' from species '*coli*'. The letter 'R' is derived from the name of strain and Roman numbers following the names indicate the order in whichthe enzymes were isolated from that strain of bacteria.

Palindromic nucleotide sequences

- The palindrome in DNA is a sequence of base pairs that reads same on the two strands when orientation of is kept the same. For example, the following sequences reads the same on the two strands in 5' 3' direction. This is also true if read in the 3' 5' direction.
- 5' —— GAATTC —— 3'
- 3' —— CTTAAG —— 5'
- Restriction enzymes cut the strand of DNA a little away from the center of the palindrome sites, but between the same two bases on the oppositestrands.

Steps in formation of recombinant DNA

- Each restriction endonuclease recognises a specific palindromic nucleotide sequences in the DNA.
- Once restriction enzymes finds its specific recognition sequence, it will bind to the DNA and cut each of the two strands of the double helix at specific points in their sugar -phosphate backbones



11.1 Steps in formation of recombinant DNA by action of restriction endonuclease enzyme - EcoRI

- This leaves single stranded portions at the ends. There are overhanging stretches called sticky ends on each strand.
- **The sticky ends**: These are named so because they form hydrogen bonds with their complementary cut counterparts. This stickiness of the ends facilitates the action of the enzyme DNA ligase.
- Restriction endonucleases are used in genetic engineering to form 'recombinant' molecules of DNA, which are composed of DNA from different sources/genomes.

Diagrammatic representation of rDNA Technology

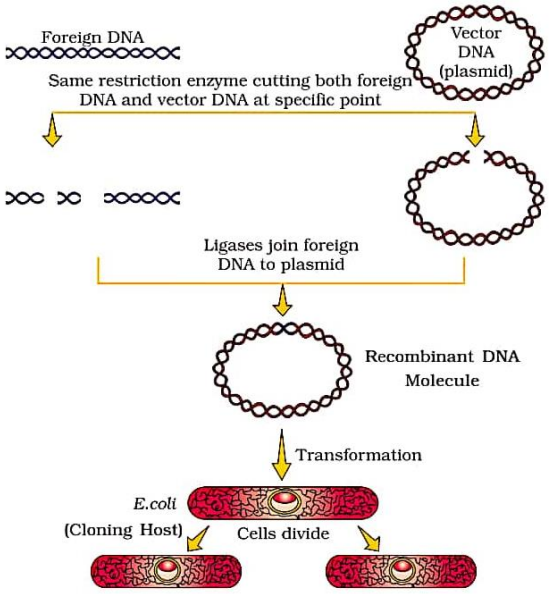


Figure 11.2 Diagrammatic representation of recombinant DNA technology

Separation and isolation of DNA fragments

Gel electrophoresis

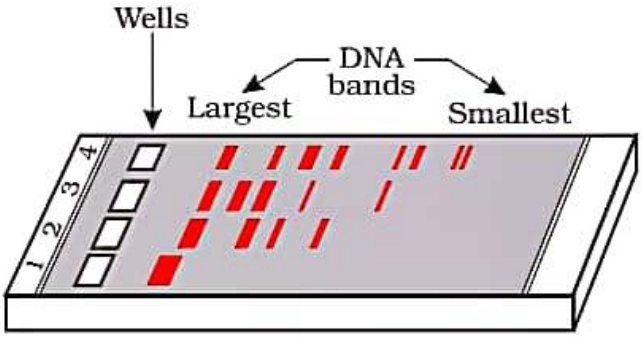


Figure 11.3 A typical agarose gel electrophoresis showing migration of undigested (lane 1) and digested set of DNA fragments (lane 2 to 4)

- The cutting of DNA byrestriction endonucleases results in the fragments of DNA. These fragmentscan be separated by a technique known as **gel electrophoresis**in an agrose medium.

- SinceDNA fragments are negatively charged molecules they can be separatedby forcing them to move towards the anode under an electric field througha medium/matrix.
- The most commonly used matrix is agarosewhich is a natural polymer extracted from sea weeds. The DNA fragmentsseparate (resolve) according to their size through sieving effect providedby the agarose gel.
- Hence, the smaller the fragment size, the farther itmoves.
- The separated DNA fragments can bevisualised only after staining the DNAwith a compound known as ethidiumbromide followed by exposure to UVradiation.
- Brightorange coloured bands of DNA in aethidium bromide stained gelexposed to UV light.
- Theseparated bands of DNA are cut outfrom the agarose gel and extractedfrom the gel piece. This step is knownas elution.
- The DNA fragmentspurified in this way are used inconstructing recombinant DNA byjoining them with cloning vectors.

Definition of Cloning Vectors

- You know that plasmids and bacteriophages have the ability to replicate within bacterial cells independent of the control of chromosomal DNA. Bacteriophages because of their high number per cell, have very high copy numbers of their genome within the bacterial cells. Some plasmids may have only one or two copies per cell whereas others may have 15-100 copies per cell. Their numbers can go even higher. If we are able to link an alien piece of DNA with bacteriophage or plasmid DNA, we can multiply its numbers equal to the copy number of the plasmid or bacteriophage. Vectors used at present, are engineered in such a way that they help easy linking of foreign DNA and selection of recombinants from non-recombinants.

Structure of cloning vector

Vector pBR 322

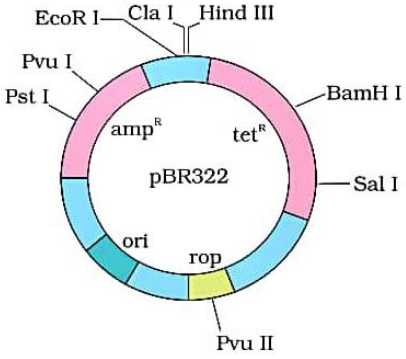


Figure 11.4 *E. coli* cloning vector pBR322 showing restriction sites (*Hind* III, *Eco*R I, *Bam*H I, *Sal* I, *Pvu* II, *Pst* I, *Cla* I), ori and antibiotic resistance genes (amp^R and tet^R). rop codes for the proteins involved in the replication of the plasmid.

- The following are the features that are required to facilitate cloninginto a vector.

Origin of replication (ori) :

- This is a sequence from where replication starts and any piece of DNA when linked to this sequence can be made to replicate with in the host cells.
- This sequence is also responsible for controlling the copy number of the linked DNA.
- So,if one wants to recover many copies of the target DNA it should becloned in a vector whose origin support high copy number.

Selectable marker :

- In addition to 'ori', the vector requires aselectable marker, which helps in identifying and eliminating nontransformantsand selectively permitting the growth of thetransformants.
- Transformation is a procedure through which apiece of DNA is introduced in a host bacterium. Normally, the genes encodingresistance to antibiotics such as ampicillin, chloramphenicol,tetracycline or kanamycin, etc., are considered useful selectablemarkers for E. coli.
- The normal E. coli cells do not carry resistance against any of these antibiotics.

Cloning sites:

- In order to link thealien DNA, the vector needs to havevery few, preferably single,recognition sites for the commonly used restriction enzymes. Presence of more than one recognition sites within the vector will generate several fragments, which will complicate the gene cloning.
- The ligation of alien DNA is carried out at restriction site present in one of the two antibiotic resistance genes. Forexample, one can ligate a foreign DNA at the BamH I site of tetracycline resistance gene in the vector pBR322.
- The recombinant plasmids will losetetracycline resistance due to insertionof foreign DNA but can still be selectedout from non-recombinant ones by plating the transformants on tetracycline containing medium.
- Transformants growing onampicillin containing medium are then transferred on a medium containing tetracycline. The recombinants will grow in ampicillin containing medium but not on that containing tetracycline. But,non- recombinants will grow on the medium containing both theantibiotics.
- In this case, one antibiotic resistance gene helps inselecting the transformants, whereas the other antibiotic resistance. gene gets 'inactivated due to insertion' of alien DNA, and helps inselection of recombinants.
- Selection of recombinants due to inactivation of antibiotics is acumbersome procedure because it requires simultaneous platingon two plates having different antibiotics.
- Therefore,alternativeselectable markers have been developed which differentiate recombinants from non-recombinants on the basis of their abilityto produce colour in the presence of a **chromogenic substrat**e.

- In this, a recombinant DNA is inserted within the coding sequence ofan enzyme, β-galactosidase. This results into inactivation of thegene for synthesis of this enzyme, which is referred to as **insertionalinactivation**.
- The presence of a chromogenic substrate gives bluecoloured colonies if the plasmid in the bacteria does not have aninsert. Presence of insert results into insertional inactivation of theb-galactosidase gene and the colonies do not produce any colour,these are identified as recombinant colonies.

Vectors for cloning genes in plants: T-DNA

- Transferring genes into plants and animals from bacteria and viruses which have been known this for ages – how to deliver genes to transform eukaryotic cells and force them to do what the bacteria or viruses want.
- *Agrobacterium tumifaciens*, a pathogen of several dicot plants is able to deliver a piece of DNA known as 'T-DNA' to transform normal plant cells into a tumor and direct these tumor cells to produce the chemicals required by the pathogen.
- The tumor inducing (Ti) plasmid of *Agrobacterium tumifaciens* has now been modified into a cloning vector which is no more pathogenic to the plants but is still able to use the mechanisms to deliver genes of our interest into a variety of plants.

Vectors for cloning genes in animals: Retro virus

- Retroviruses in animals have the ability to transform normal cells into cancerous cells. A better understanding of the art of delivering genes by pathogens in their eukaryotic hosts has generated knowledge to transform these tools of pathogens into useful vectors for delivering genes of interest to humans.
- Retroviruses are **disarmed** and are now used to deliver desirable genes into animal cells. So, once a gene or a DNA fragment has been ligated into a suitable vector it is transferred into a bacterial,plant or animal host where it multiplies.

Gene delivery

Transfer of plasmid into a bacterium

- DNA is a hydrophilic molecule, it cannot pass through cell membranes. In order to force bacteria to take up the plasmid, thebacterial cells must first be made 'competent' to take up DNA.
- This isdone by treating them with a specific concentration of a divalent cation,such as calcium, which increases the efficiency with which DNA entersthe bacterium through pores in its cell wall.
- Recombinant DNA can thenbe forced into such cells by incubating the cells with recombinant DNAon ice, followed by placing them briefly at 42^0C (heat shock), and thenputting them back on ice. This enables the bacteria to take up therecombinant DNA.

Physical basis of gene delivery

- **Micro-injection** method recombinant DNA is directly injectedinto the nucleus of an animal cell. (Fig. to be downloaded from net)
- **Gene gun method**: suitable for plants,cells are bombarded with high velocity micro-particles of gold or tungstencoated with DNA in a method known as biolistics or gene gun.

Vector mediated:

- 'Disarmed pathogen' vectors, which when allowed toinfect the cell, transfer the recombinant DNA into the host.
- For example T-DNA, retro virus etc.

Isolation of genetic material

- Nucleic acid is the genetic material of all organisms without exception. In majority of organisms this is deoxyribonucleic acid or DNA. In order to cut the DNA with restriction enzymes, it needs to be in pure form, free from other macro-molecules.
- As DNA is enclosed within the membranes. The membrane need to be brokento open up the cell to release DNA along with other macromolecules such as RNA, proteins, polysaccharides and also lipids.
- This can be achieved by treating the bacterial cells/plant or animal tissue with enzymes such as lysozyme (bacteria), cellulase (plant cells), chitinase (fungus).

Purification of DNA

- The Genomic DNA thus obtained contains impurities in form of RNA, protein etc.
- RNA can be removed bytreatment with ribonuclease whereas proteins can be removed bytreatment with protease.
- Other molecules can be removed by appropriatetreatments and purified.
- DNA ultimately precipitates out after the additionof chilled ethanol. This can be seen as collection of fine threads in thesuspension.
- DNA that separates out can be removed by spooling.

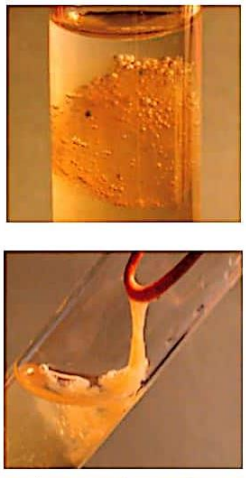


Figure 11.5 DNA that separates out can be removed by spooling

Amplification of Gene of Interest by PCR

- PCR was invented in 1983 by Kary Mullis. It is fundamental to much of genetic testing including analysis of ancient samples of DNA and identification of infectious agents.
- PCR stands for Polymerase Chain Reaction. In this reaction, multiple copies of the gene of interest is synthesized.
- Synthesis of gene of interest is carried out*in vitro*by using two sets of primers (small chemically synthesised oligonucleotides that are complementary to the regions of DNA) and the enzyme DNA polymerase.
- The enzyme extends the primers using the nucleotides provided in thereaction and the genomic DNA as template. If the process of replicationof DNA is repeated many times, the segment of DNA can be amplifiedto approximately billion times, i.e., 1 billion copies are made.
- Suchrepeated amplification is achieved by the use of a thermostable DNApolymerase (Taq polymerase)isolated from a bacterium, *Thermus aquaticus*, whichremain active during the high temperature induced denaturation ofdouble stranded DNA. The amplified fragment if desired can now beused to ligate with a vector for further cloning.

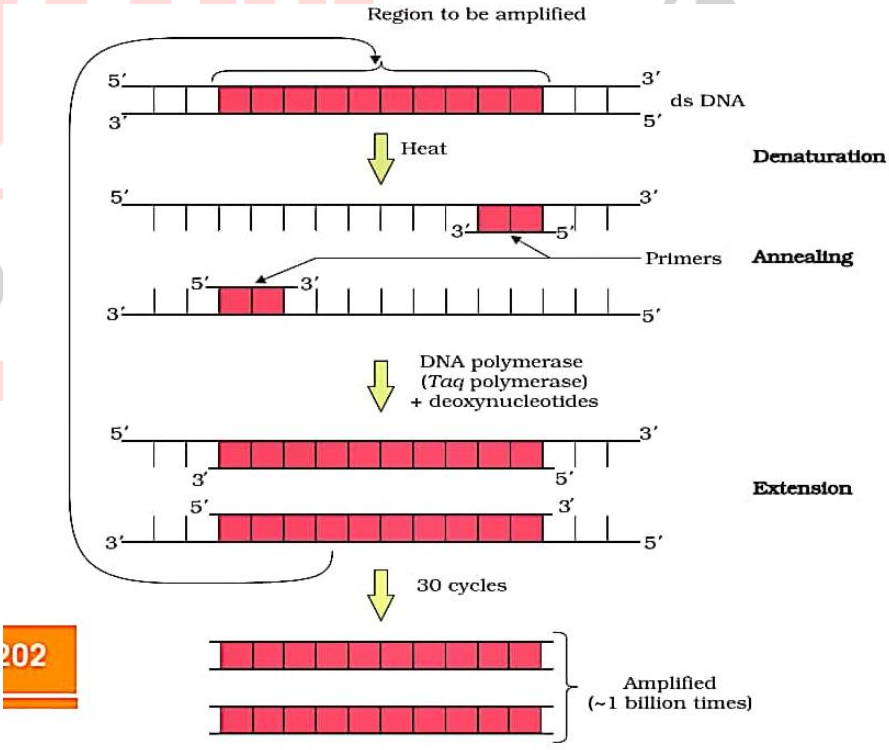


Figure 11.6 Polymerase chain reaction (PCR) : Each cycle has three steps: (i) Denaturation; (ii) Primer annealing; and (iii) Extension of primers

Method of PCR

- Each cycle of PCR has three steps: (i) Denaturation;(ii) Primer annealing; and (iii) Extension of primers

Denaturation

- The majority of PCR methods rely on thermal cycling. Thermal cycling exposes reactants to repeated cycles of heating and cooling to permit different temperature-dependent reactions – specifically, DNA melting and enzyme-driven DNA replication.
- In the first step of PCR, the two strands of the DNA double helix are physically separated at a high temperature in a process called Nucleic acid denaturation.
- This step is the first regular cycling event and consists of heating the reaction chamber to 94–98 °C (201–208 °F) for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.
- The two DNA strands then become templates for DNA polymerase to enzymatically assemble a new DNA strand from free nucleotides, the building blocks of DNA.

Primer annealing

- In the second step, the temperature is lowered and the primers bind to the complementary sequences of DNA, this is called as annealing.
- In this step, the reaction temperature is lowered to 50–65 °C (122–149 °F) for 20–40 seconds, allowing annealing of the primers to each of the single-stranded DNA templates.
- Two different primers are typically included in the reaction mixture: one for each of the two single-stranded complements containing the target region.
- The primers are single-stranded sequences themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3' end of each strand.

Extension of primers

- The optimum activity temperature for the thermostable DNA polymerase of Taq polymerase is approximately 75–80 °C. In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxy group at the end of the nascent (elongating) DNA strand.
- Under optimal conditions at each extension/elongation step, the number of DNA target sequences is doubled. With each successive cycle, the original template strands plus all newly generated strands become template strands for the next round of elongation, leading to exponential (geometric) amplification of the specific DNA target region.

Expression of the foreign gene

- When you insert a piece of alien DNA into a cloning vector and transfer itinto a bacterial, plant or animal cell, the alien DNA gets multiplied. Inalmost all recombinant technologies, the ultimate aim is to produce adesirable protein. Hence, there is a need for the recombinant DNA to beexpressed. The foreign gene gets expressed under appropriate

conditions.The expression of foreign genes in host cells involve understanding manytechnical details.

- After having cloned the gene of interest and having optimised theconditions to induce the expression of the target protein, one has toconsider producing it on a large scale. Can you think of any reasonwhy there is a need for large-scale production? If any protein encodinggene is expressed in a heterologous host, it is called a recombinant protein. The cells harbouring cloned genes of interest may be grown on a small scale in the laboratory.
- The cultures may be used forextracting the desired protein and then purifying it by using differentseparation techniques.The cells can also be multiplied in a continuous culture system whereinthe used medium is drained out from one side while fresh medium isadded from the other to maintain the cells in their physiologically most active log/exponential phase. This type of culturing method produces alarger biomass leading to higher yields of desired protein.

Obtaining recombinant protein

Bioreactors

- Bioreactors are large vessels in which raw materials are biologically converted into specific products, individual enzymes, etc., using microbial plant, animal or human cells.
- It produces biological products in large scale, where large volumes (100-1000 litres) of culture can be processed.
- A bioreactor provides the optimal conditions for achieving the desired product by providing optimum growth conditions: temperature, pH, substrate, salts, vitamins, oxygen.

Simple stirred-tank bioreactor and Sparged stirred-tank bioreactor

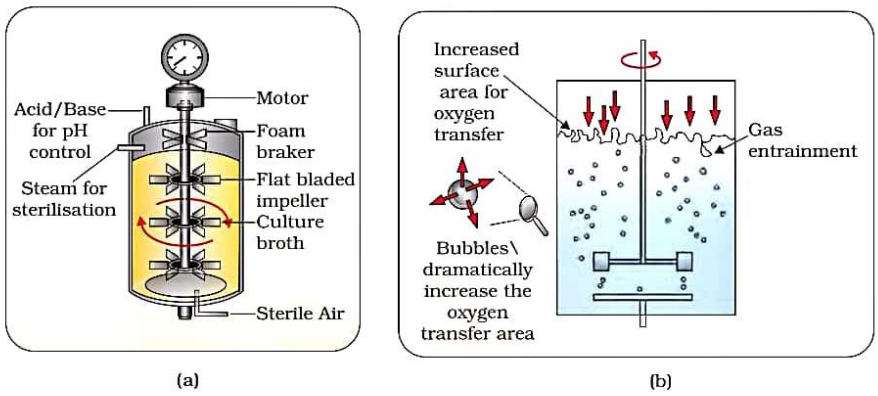


Figure 11.7 (a) Simple stirred-tank bioreactor; (b) Sparged stirred-tank bioreactor through which sterile air bubbles are sparged

- **A stirred-tank reactor** is usually cylindrical or with a curved base tofacilitate the mixing of the reactor contents. The stirrer facilitates evenmixing and oxygen availability throughout the bioreactor.
- Alternativelyair can be bubbled through the reactor. The bioreactor has an agitator system, an oxygen deliverysystem and a foam control system, a temperature control system,

pHcontrol system and sampling ports so that small volumes of the culturecan be withdrawn periodically.

- **Sparged tank bioreactor: The reactor** has all the features to that of simple tank bioreactor. The basic difference is that sterile air bubbles are sparged through the bio reactor. Bubbles increase the oxygen transfer area.

Downstream processing

- After completion of the biosynthetic stage, the biological product has to be subjectedthrough a series of processes before it is ready for marketing as a finished product.
- The processes include separation and purification, which arecollectively referred to as downstream processing.
- The product has to beformulated with suitable preservatives. Such formulation has to undergothorough clinical trials as in case of drugs. Strict quality control testingfor each product is carried out to ensure the quality of the product.

Sl No.	Terms	Explanation
1	Biotechnology	'The integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services'
2	EFB	The European Federation of Biotechnology
3	Genetic engineering	Techniques to alter the chemistry of genetic material (DNA and RNA), to introduce these into host organisms and thus change the phenotype of the host organism
4	Bioprocess engineering	Maintenance of sterile (microbial contamination-free) ambience in chemical engineering processes to enable growth of only the desired microbe/eukaryotic cell in large quantities for the manufacture of biotechnological products like antibiotics, vaccines, enzymes, etc.
5	gene cloning	an alien DNA is linked with the origin of replication, so that, this alien piece of DNA can replicate and multiply itself in the host organism
6	gene transfer	Transfer of genetic material between unicellular or multicellular organisms other than transmission of DNA from parent to offspring
7	plasmid	(autonomously replicating circular extra-chromosomal DNA)
8	restriction enzymes	'molecular scissors'/The enzymes responsible for restricting the growthof bacteriophage in bacteria cells
9	Restriction Endonuclease	The restriction enzyme that cuts the DNA in between two specific nucleotides .
10	Restriction Exonuclease	Exonucleases remove nucleotides from the ends of the DNA

11	Vectors	A plasmid or a virus which is used as a vehicle to transfer the gene of interest into the host cell
12	Recognition sequence	The specific sequence of nucleotides recognized by a restriction enzyme
13	Palindromic nucleotide sequences	DNA is a sequence of base pairs that reads same on the two strands when orientation of is kept the same
14	Gel electrophoresis	Separation of DNA fragments digested by restriction endonuclease in an agrose medium
15	DNA fragments	Fragments of DNA produced when treated with restriction enzymes
16	Recombinants	Organsims produced by transfer of alien DNA in the process creating sequences that would not otherwise be found in the genome
17	Non-recombinants	An organism which genome has not been changed by gene transfer
18	Selectable marker	Are the genes in a vector which helps in identifying and eliminating nontransformantsand selectively permitting the growth of thetransformants
19	Transformation	It is a procedure through which apiece of DNA is introduced in a host bacterium.
20	Transformants	A cell or an organism that has taken up an additional alien DNA
21	Nontransformants	A cell or an organism that has not taken up an alien DNA
22	Cloning site	Is a short segment of DNA which contains restriction sites
23	pBR322	*E. coli* cloning vector
24	Insertional inactivation	Insertion of recombinant DNA in a vector which results into inactivation of thegene for synthesis of this enzyme.
25	T-DNA	A piece of DNA found in Ti-plasmid known to deliver into plant cells to transform normal cells into a tumor and direct these tumor cells to produce thechemicals required by the pathogen.
26	Ti- plasmid	Tumor inducing plasmid of *Agrobacterium tumifaciens*
27	Gene delivery	Transfer a gene of interest into the host cell/organism.
28	Biolistic method	Cells are bombarded with high velocity micro-particles of gold or tungstencoated with alien DNA
29	Micro-injection method	To introduce alien DNA (recombinant DNA) into host cells by directly injecting it into the nucleus of an animal cell
30	Lysozyme	Enzyme that digests bacteria cell wall
31	Cellulase	Enzyme that digests plant cells
32	Chitinase	Enzyme that digests fungi cell wall
33	Spooling	The technique of separating DNA sedimentfrom the tube.
34	PCR	Stands for Polymerase Chain Reaction

35	Denaturation	Formation of dsDNA to ssDNA by heating
36	Primer annealing	Is the binding of primer to 3' of the ssDNA
37	Extension of primers	Is the chain elongation by *Taq polymerase*
38	*Taq* polymerase	Thermostable DNA polymerase isolated from a bacterium, *Thermus aquaticus*
39	Recombinant protein	Any protein encoding gene expressed in a heterologous host
40	Bioreactors	A bioreactors is a vessels in which raw materials arebiologically converted into specific products, individual enzymes, etc.,using microbial, plant, animal or human cells
41	Downstream Processing	Refers to the recovery and purification of biosynthetic products obtained from a broth culture.