gene gets 'inactivated due to insertion' of alien DNA, and helps in selection of recombinants.

Selection of recombinants due to inactivation of antibiotics is a cumbersome procedure because it requires simultaneous plating on two plates having different antibiotics. Therefore, alternative selectable markers have been developed which differentiate recombinants from non-recombinants on the basis of their ability to produce colour in the presence of a chromogenic substrate. In this, a recombinant DNA is inserted within the coding sequence of an enzyme, β-galactosidase. This results into inactivation of the enzyme, which is referred to as **insertional inactivation**. The presence of a chromogenic substrate gives blue coloured colonies if the plasmid in the bacteria does not have an insert. Presence of insert results into insertional inactivation of the â-galactosidase and the colonies do not produce any colour, these are identified as recombinant colonies.

(iv) ***Vectors for cloning genes in plants and animals :*** You may be surprised to know that we have learnt the lesson of transferring genes into plants and animals from bacteria and viruses which have known this for ages – how to deliver genes to transform eukaryotic cells and force them to do what the bacteria or viruses want. For example, *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. Similarly, 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. 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. Similarly, retroviruses have also been 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).

11.2.3 Competent Host (For Transformation with Recombinant DNA)

Since DNA is a hydrophilic molecule, it cannot pass through cell membranes. *Why*? In order to force bacteria to take up the plasmid, the bacterial cells must first be made 'competent' to take up DNA. This is done by treating them with a specific concentration of a divalent cation, such as calcium, which increases the efficiency with which DNA enters

the bacterium through pores in its cell wall. Recombinant DNA can then be forced into such cells by incubating the cells with recombinant DNA on ice, followed by placing them briefly at 42°C (heat shock), and then putting them back on ice. This enables the bacteria to take up the recombinant DNA.

This is not the only way to introduce alien DNA into host cells. In a method known as **micro-injection**, recombinant DNA is directly injected into the nucleus of an animal cell. In another method, suitable for plants, cells are bombarded with high velocity micro-particles of gold or tungsten coated with DNA in a method known as **biolistics** or **gene gun**. And the last method uses 'disarmed pathogen' vectors, which when allowed to infect the cell, transfer the recombinant DNA into the host.

Now that we have learnt about the tools for constructing recombinant DNA, let us discuss the processes facilitating recombinant DNA technology.

11.3 Processes of Recombinant DNA Technology

Recombinant DNA technology involves several steps in specific sequence such as isolation of DNA, fragmentation of DNA by restriction endonucleases, isolation of a desired DNA fragment, ligation of the DNA fragment into a vector, transferring the recombinant DNA into the host, culturing the host cells in a medium at large scale and extraction of the desired product. Let us examine each of these steps in some details.

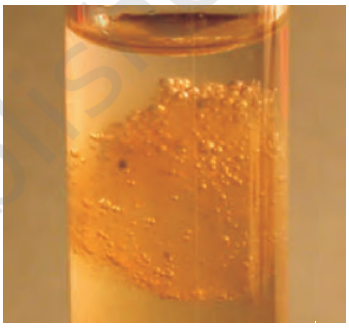

Figure 11.5 DNA that separates out can be removed by spooling

11.3.1 Isolation of the Genetic Material (DNA)

Recall that 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. Since the DNA is enclosed within the membranes, we have to break the cell open 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). You know that genes are located on long molecules of DNA interwined with proteins such as histones. The RNA can be removed by treatment with ribonuclease whereas proteins can be removed by treatment with protease. Other molecules can be removed by appropriate treatments and purified DNA ultimately precipitates out after the addition of chilled ethanol. This can be seen as collection of fine threads in the suspension (Figure 11.5).

11.3.2 Cutting of DNA at Specific Locations

Restriction enzyme digestions are performed by incubating purified DNA molecules with the restriction enzyme, at the optimal conditions for that specific enzyme. Agarose gel electrophoresis is employed to check the progression of a restriction enzyme digestion. DNA is a negatively charged molecule, hence it moves towards the positive electrode (anode) (Figure 11.3). The process is repeated with the vector DNA also.

The joining of DNA involves several processes. After having cut the source DNA as well as the vector DNA with a specific restriction enzyme, the cut out 'gene of interest' from the source DNA and the cut vector with space are mixed and ligase is added. This results in the preparation of recombinant DNA.

11.3.3 Amplification of Gene of Interest using PCR

PCR stands for **Polymerase Chain Reaction**. In this reaction, multiple copies of the gene (or DNA) of interest is synthesised *in vitro* using two

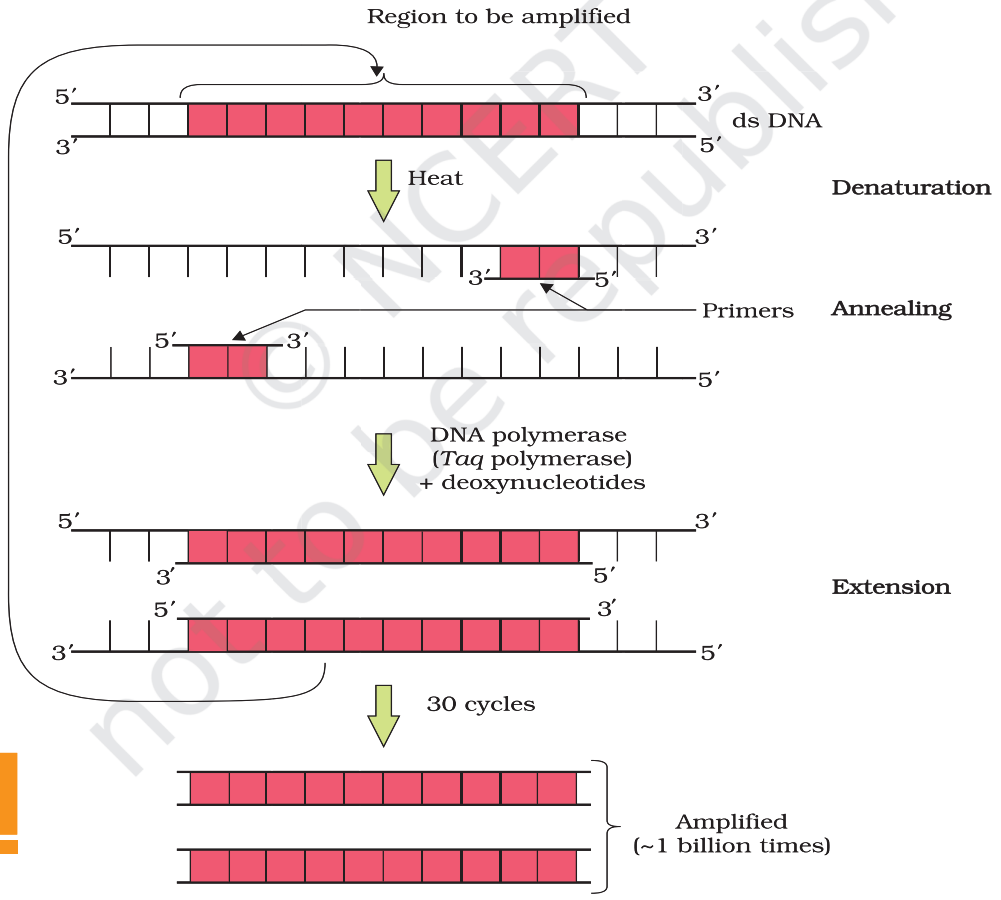

Figure 11.6 Polymerase chain reaction (PCR) : Each cycle has three steps: (i) Denaturation; (ii) Primer annealing; and (iii) Extension of primers

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 the reaction and the genomic DNA as template. If the process of replication of DNA is repeated many times, the segment of DNA can be amplified to approximately billion times, i.e., 1 billion copies are made. Such repeated amplification is achieved by the use of a thermostable DNA polymerase (isolated from a bacterium, *Thermus aquaticus*), which remain active during the high temperature induced denaturation of double stranded DNA. The amplified fragment if desired can now be used to ligate with a vector for further cloning (Figure11.6).

11.3.4 Insertion of Recombinant DNA into the Host Cell/Organism

There are several methods of introducing the ligated DNA into recipient cells. Recipient cells after making them 'competent' to receive, take up DNA present in its surrounding. So, if a recombinant DNA bearing gene for resistance to an antibiotic (e.g., ampicillin) is transferred into *E. coli* cells, the host cells become transformed into ampicillin-resistant cells. If we spread the transformed cells on agar plates containing ampicillin, only transformants will grow, untransformed recipient cells will die. Since, due to ampicillin resistance gene, one is able to select a transformed cell in the presence of ampicillin. The ampicillin resistance gene in this case is called a **selectable marker**.

11.3.5 Obtaining the Foreign Gene Product

When you insert a piece of alien DNA into a cloning vector and transfer it into a bacterial, plant or animal cell, the alien DNA gets multiplied. In almost all recombinant technologies, the ultimate aim is to produce a desirable protein. Hence, there is a need for the recombinant DNA to be expressed. The foreign gene gets expressed under appropriate conditions. The expression of foreign genes in host cells involve understanding many technical details.

After having cloned the gene of interest and having optimised the conditions to induce the expression of the target protein, one has to consider producing it on a large scale. *Can you think of any reason why there is a need for large-scale production*? If any protein encoding gene 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 for extracting the desired protein and then purifying it by using different separation techniques.

The cells can also be multiplied in a continuous culture system wherein the used medium is drained out from one side while fresh medium is added from the other to maintain the cells in their physiologically most

active log/exponential phase. This type of culturing method produces a larger biomass leading to higher yields of desired protein.

Small volume cultures cannot yield appreciable quantities of products. To produce in large quantities, the development of **bioreactors**, where large volumes (100-1000 litres) of culture can be processed, was required. Thus, bioreactors can be thought of as vessels in which raw materials are biologically converted into specific products, individual enzymes, etc., using microbial plant, animal or human cells. A bioreactor provides the optimal conditions for achieving the desired product by providing optimum growth conditions (temperature, pH, substrate, salts, vitamins, oxygen).

The most commonly used bioreactors are of stirring type, which are shown in Figure 11.7.

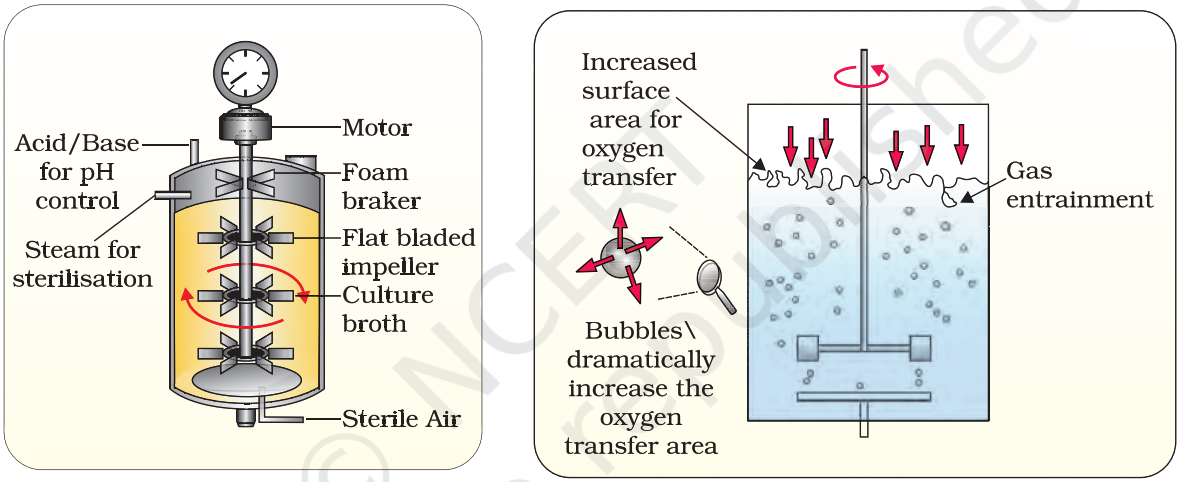

Figure 11.7 (a) Simple stirred-tank bioreactor; (b) Sparged stirred-tank bioreactor through which sterile air bubbles are sparged

A stirred-tank reactor is usually cylindrical or with a curved base to facilitate the mixing of the reactor contents. The stirrer facilitates even mixing and oxygen availability throughout the bioreactor. Alternatively air can be bubbled through the reactor. If you look at the figure closely you will see that the bioreactor has an agitator system, an oxygen delivery system and a foam control system, a temperature control system, pH control system and sampling ports so that small volumes of the culture can be withdrawn periodically.

11.3.6 Downstream Processing

After completion of the biosynthetic stage, the product has to be subjected through a series of processes before it is ready for marketing as a finished