Chapter 18 MICROBIAL CULTURE AND APPLICATIONS

In This Chapter

18.1 INTRODUCTION

Microbial populations dominate the biosphere in terms of metabolic impact and numbers. Among the various types of microbes, prokaryotes are the most pervasive lifeform on the planet, often tolerating extremes in pH, temperature, salt concentration, etc. Metabolic diversity is greater among prokaryotes than all eukaryotes combined. Men have long been utilizing the bacterial and yeast and fungal population for the manufacturing of various chemicals, biochemicals, antibiotics, beverages, etc. Production of antibiotics,

alcohols, vinegar, amino acids, vitamins, therapeutic antibodies, acetone and other solvents, and recombinant proteins is accomplished by the large-scale cultivation of microbial cells such as bacteria, algae, yeast, and fungus on industrial scale. In all these industrial applications the metabolic activities or the biochemical pathways are used for the production of specific chemicals with the consumption of the substrates or a carbon source such as sucrose. Here, the microbial culture acts as a factory, where the substrate is the raw material. It is converted into the product and secreted into the media. The product can be recovered from the media with a process called downstream processing. There is a limitation for a single cell to convert the raw material into products in a given period of time. It is possible to calculate the rate of product formation by a single cell under a specific metabolic condition, if we know the quantity of product formed over a period of time and the number of cells in the culture. If we want to produce a specific quantity of the product over a period of time it is possible to calculate the number of bacterial or microbial cells required to operate the bioprocess on an industrial scale.

Like any other chemical equation this microbial-mediated biotransformation can also be considered a chemical reaction and can be expressed by a chemical equation:

$$C_wH_xO_yN_z + aO_2 + bH_gO_hN \longrightarrow cCHaObNd + dCO_2 + eH_2O$$

Even though the microbial conversion of a substrate can be compared to a chemical reaction, where the reactant is converted into a product, the efficiency of conversion will be comparatively less. A chemical reaction requires an appropriate temperature, pressure, pH, and solvent system for maximum product output. The microbial system also has to be provided with the optimum environmental and nutritional conditions such as temperature, pH, and the correct substrate for converting it into the product. The efficiency of microbial conversion of substrate into product is comparatively less because a major part of the metabolic energy is utilized for the generation of biomass by cell growth and multiplication.

18.2 MICROBIAL CULTURE TECHNIQUES

As stated previously, microbial cultures should be provided with the required chemical and physical environment for proper multiplication and physiological state, so that the cells can carry out the required bioconversion satisfactorily. The chemical environment of a microbial cell is its nutritional conditions in which it is growing. It also includes the correct pH and temperature.

Nutrients for Microbial Culture

Like any other living system, microorganisms also require a source of energy, carbon, nitrogen, oxygen, iron and other minerals, micronutrients, and water for growth, and multiplication. All these nutrients that are essential for the growth and multiplication of microbial organisms are supplied in the form of nutrient media. For laboratory-scale cultivation we may use certain costly media components, but for industrial purposes they should be economical and readily available. The media that we use for growing microbes may be synthetic, semi-synthetic, or completely natural. If the nutritive components of the media are not of natural origin, such nutritive media are known as **synthetic media**, which we can synthesize in the laboratory following certain recipes, mixing the required salts, minerals, and carbon source. There are a large number of commercially-available nutrient media, which contain both salts and minerals. Such nutrient media are known as **semi-synthetic media.** For example, commercially available nutrient broth, trypticase soya broth (TSB), brain-heart infusion (BHI) broth, yeast extract, potato dextrose agar, casein digest, etc., are some examples of semi-synthetic media. For laboratory-scale cultivation of bacteria and other microorganisms, these synthetic or semi-synthetic media are preferred, but for industrial-scale cultivation these media are not recommended from an economical point of view. For commercial purposes, the recommended media should be cheap and available year round. The following are the minimum components required in a microbial medium for cultivation of microbes in a laboratory:

Carbon source. A simple carbon source, which is simple to use and easily available, can be used. Sugars such as glucose, lactose, sucrose, and complex polysaccharides such as starch, glycogen cellulose, a mixture of various carbohydrates, and other compounds such as cereal grain powders, cane molasses, etc., are usually used as carbon sources in microbial culture media. The main purpose of the carbon source is to provide energy and carbon skeleton for the synthesis of various other biological compounds.

Nitrogen sources. The major types of nitrogen sources used in culture media are ammonium salts, urea, animal tissue extracts, amino acid mixtures, and plant-tissue extracts.

Microelements or trace elements. Elements required in small amounts or in traces are to be added into the medium as salts in required amounts. The elements such as copper, cobalt, iron, zinc, manganese, magnesium, etc., are the microelements.

Growth factors. Growth factors are certain organic compounds that are essential for the growth and multiplication of cells, but cannot be synthesized by the cells. Such compounds should be supplemented in the medium. Certain amino acids and vitamins are also included in this category.

Anti-foams. This is not a nutritive component of the media. Media rich in nutritive components such as starch, protein, and other organic material and also the proteins and other products secreted by the growing cells can result in excessive foaming while the culture media is agitated for aeration. To prevent the formation of foam some anti-foaming agents are included in the media. Certain types of fatty acids such as olive oil and sunflower oil and silicones are commonly used in cell cultures as anti-foam agents.

Energy sources. The carbon sources used in culture media such as carbohydrates, sugars, proteins, lipids, etc., can work as energy sources for the growth and metabolism of the microbial cells.

Water. Water is the base of any culture media, whether it is liquid or solid. In solid culture media such as the media of solid state fermentation or agar media the quantity of water is comparatively less than liquid media. In laboratory experiments, single-distilled water or double-distilled water is usually used. But in large-scale microbial cultivation for industrial purposes, the pH and the dissolved salts present should be considered when formulating the media requirements and its concentration. Water is also required for a large number of other services in the laboratory such as cooling, heating, steaming, etc. Therefore, any laboratory should be provided with a source of clean water of consistent quality.

Culture Procedures

1. **Sterilization.** The media and culture vessel have to be sterilized to prevent the growth of unwanted microorganisms and thus contamination. If laboratory-scale experiments are carried out in 100 to 1,000 ml flasks, or in lesser volumes such as 50 ml or 10 ml, the media along with the culture flasks or vials can be steam-sterilized with an autoclave. Depending on the quantity of the materials autoclaved, the sterilization can be carried out alternatively in a pressure cooker of convenient size. Steam-sterilization with an autoclave or pressure cooker is carried out at 120°C for 15 to 20 minutes under 15 psi pressure.

   When microbes are cultivated in a fermentor for large-scale operation, it is convenient to sterilize the fermentor as a whole with or without media. Media may be sterilized separately or *in situ*, in the fermentor itself. Steam is used for the sterilization of the media and fermentor, by passing the steam through the sterilization jacket or the coil around the fermentor. When the fermentor is sterilized without media in it, steam can be sparged into the vessel through all openings, allowing it to exit very slowly. Sparging is a process by which sterile air or steam is allowed to pass through the medium in the vessel with the help of a sparging device placed at the bottom of the fermentor. The steam pressure is held at 15 psi for 20 to 30 minutes while circulating or holding the steam within the vessel or in the jacket (Figure 18.1).

2. **Environment for microbial growth.** The nutrient composition of the medium, the ionic concentration of salts, pH, and temperature influence the growth of microorganisms in the culture and its metabolic state. Most of the bacteria grow at neutral pH, where as yeast and fungi prefer acidic pH. Similarly, different organisms prefer different optimum temperatures for active growth and multiplication. The optimum temperature has to be maintained in the culture with the help of an incubator in the case of small-scale cultures and circulating water of the appropriate temperature through the jacket of the fermentor.
3. **Aeration and mixing.** Mixing of the broth is essential for the uniform distribution of the nutrients and the microbial population in the culture. Aeration is needed for the easy gas exchange between the medium and the environment. Aerated medium will be rich in oxygen. Aeration and mixing can be easily achieved by shaking the medium on a shaker in the case of small-scale cultures (shake flasks cultures). In large-scale cultivation in bioreactors the transfer of oxygen to organisms is very difficult because it requires proper mixing. In fermentors, the proper mixing of cells, media components, and oxygen is achieved by stirring the medium with the help of a mechanical stirrer with baffles attached to it. Baffles help in maintaining turbulence. Microbial-free air passed through the media ensures proper aeration, and this forced aeration also helps in the mixing of media, cells, and oxygen.

Microbial Culture Equipment

In the laboratory, microbial cells can be grown in tubes and vials, when the volume is five to ten ml, and in Erlenmeyer flasks when the volume is 100 to 1,000 ml. Improvements in the culturing of microbes can be done by making improvements in the design of the flasks and also by using shakers.

Baffle Flasks

Baffle flasks are the modified flasks for microbial cultivation, in which there are v-shaped notches or indentations in the sides of the flasks. The presence of baffles improves the efficiency of oxygen transfer and thereby the growth of microbes because the baffles increase the turbulence while the media is agitated on a shaker.

Shakers

Shakers are the special equipment designed for rotating a platform orbitaly, so that the culture flasks with media kept on the platform of the shaker will be continuously agitated. This agitation helps the medium to be homogeneous in cell-mass distribution, media components, and efficient oxygen transfer.

Fermentors

These are bioreactors used for the cultivation of microbial cells on large scale under controlled conditions for industrial purposes. This closed metallic or glass vessel has the adequate arrangement for aeration, mixing of media by agitation, temperature control, pH control, anti-foaming, control of overflow, sterilization of media and vessel, cooling, and sampling (removal of sample, while the fermentor is on). Agitation of the media in the bioreactor may be through stirring or aeration or both. This equipment is convenient for operation continuously for a number of days. The essential parts of a laboratory fermentor are given in Figure 18.1.

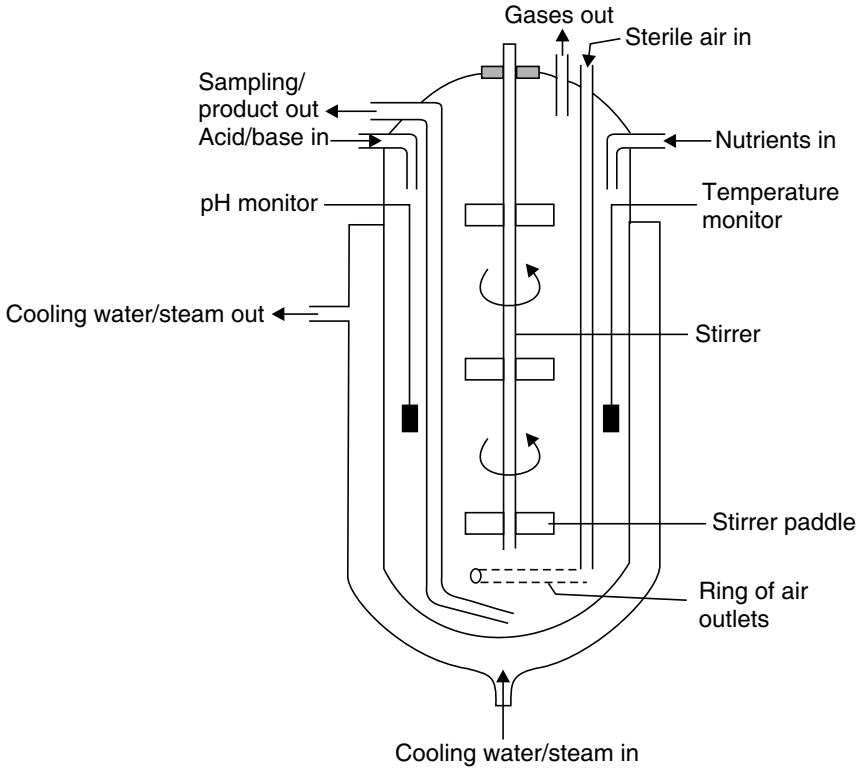

FIGURE 18.1 **Diagram sketch of a laboratory fermentor showing the essential components.**

As indicated in the figure, the bioreactors are provided with controls for monitoring and adjusting the many physical and chemical parameters such as temperature, pH, nutrient composition, foaming, etc. Maximum cell growth and product formation can be achieved by controlling these parameters that assist cell growth and metabolism leading to high output of the product. A stirred tank bioreactor is the most commonly used bioreactor for microbial cultivation, in which the microbial medium is stirred with an impeller. A high density of metabolically

active cells in the medium can result in sudden depletion of dissolved oxygen creating an anaerobic condition in the medium. This can result in serious consequences in the quality of the product or even in the type of products formed in the fermentation reaction. Similarly, the cell growth and product formation can alter pH of the medium, which can also create problems in the further growth and metabolism of the cell cultures. Rapid growth also results in the depletion of essential nutrients that directly link to the growth and metabolism that causes the production of the product. All these changes are monitored by the accessories of the fermentor or bioreactor and are accordingly indicated or rectified automatically. For example, whenever there is a change in pH from the optimum value, automatically a sufficient amount of acid or alkali is added to the media to keep the optimum pH constant. Similarly, if there is foaming in the media the sensor will detect the foam formation and accordingly, the antifoam agent is delivered into the medium to prevent the foaming.

In addition to the industrial type of bioreactors or fermentors, there are fermentors of small volumes suitable for operating in the laboratories, known as **laboratory fermentors.** These laboratory fermentors are for 10 to 100 liters of volume and are used for optimizing culture conditions and nutritional parameters for better growth of cells and production of metabolites for conducting research studies in the laboratory.

Types of Microbial Cultures

The culturing of the microbial system can be achieved in different ways. The type of culture method sometimes depends on the type of the microbial system or on the type of the product that we expect. For example, one can get two entirely different products from the same organism by changing the nutritional and other parameters or even culturing vessels.

1. **Batch culture.** This is a small-scale laboratory experiment in which a microbial culture is growing in a small volume flask. It consists of a limited volume of broth culture in a flask inoculated with the bacterial or microbial inoculum and follows a normal growth phase. It is a closed-culture system because the medium contains a limited amount of nutrients and will be consumed by the growing microorganisms for their growth and multiplication with the excretion of certain metabolites as products. In **batch cultures**, the nutrients are not renewed and the exponential growth of cells is limited to a few generations. The growth phase of the culture consists of an initial lag phase, a log phase or the exponential growth phase, and a stationary phase. During the log phase the consumption of the nutrients will be the maximum resulting in the maximum biomass output with the excretion of the product. At the stationary phase the rate of growth decreases and becomes zero. This is because at the

stationary phase the cells are exposed to a changed environment where there is only a small amount of nutrients and more cells along with the accumulation of metabolites, which may have a negative effect on the growth of the cells.

2. **Fed-batch culture.** The batch culture can be made into a semi-continuous culture or fed-batch culture by feeding it with fresh media sequentially at the end of the log phase or in the beginning of the stationary phase without removing cells. Because of this the volume of the culture will go on increasing as fresh media is added. This method is specially suited for cultures in which a high concentration of substrate is inhibitory to cell multiplication and biomass formation. In such situations the substrate can be fed at low concentrations to achieve cell growth. This method can easily produce a high cell density in the culture medium, which may not be possible in a batch fermentor or shake flask culture. This is especially important when the product formation is intracellular to achieve maximum product output per biomass.
3. **Continuous culture.** Bacterial cultures can be maintained in a state of exponential growth over long periods of time using a system of **continuous culture**, designed to relieve the conditions that stop exponential growth in batch cultures. Continuous culture, in a device called a **chemostat**, can be used to maintain a bacterial population at a constant density, a situation that is, in many ways, more similar to bacterial growth in natural environments.

   This is a very convenient method to get continuous cell growth and product formation over a long period of time. In continuous culture, the nutrient medium including the raw material is supplied at a rate that is equal to the volume of media with cells and product displaced or removed from the culture. The volume removed and the volume added is the same. In effect there is no change in the net volume as well as the chemical environment of the culture.

   In a **chemostat**, the growth chamber is connected to a reservoir of sterile medium. Once the growth is initiated, fresh medium is continuously supplied from the reservoir (Figure 18.2). The volume of fluid in the growth chamber is maintained at a constant level by some sort of overflow drain. Fresh medium is allowed to enter into the growth chamber at a rate that limits the growth of the bacteria. The bacterial cells grow (cells are formed) at the same rate at which bacterial cells (and spent medium) are removed by the overflow. The rate of addition of the fresh medium determines the rate of growth because the fresh medium always contains a limiting amount of an essential nutrient. Thus, the chemostat relieves the insufficiency of nutrients, the accumulation of toxic substances, and the accumulation of excess cells in the culture, which are the parameters that initiate the stationary phase of the growth cycle. The bacterial culture can be grown and maintained at relatively constant conditions, depending on the flow rate of the nutrients.

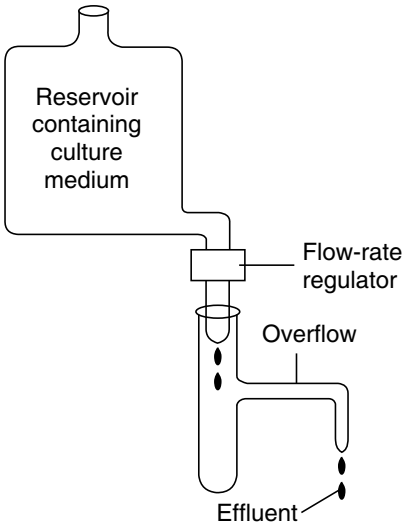

FIGURE 18.2 **Schematic diagram of a chemostat, a device used for the continuous culture of microbes. The chemostat relieves the environmental conditions that restrict growth by continuously supplying nutrients to cells and removing waste substances and spent cells from the culture medium.**

If the chemical environment is constant in a chemostat continuous culture, the cell density is constant in a turbidostat culture, which is also a continuous culture. Since the culture is fed with the fresh medium at specific rate, a steady state of growth and metabolism is achieved. At a steady state, the cell multiplication and substrate consumption for growth and product formation occur at a fixed rate. The growth rate is maintained constantly. The formation of new biomass is balanced with the removal of cells from the outlet. Continuous culture is very suitable for the production of cell biomass and products, if it is excreted into the medium. It is widely used for the production of single-cell protein from liquid effluents as a byproduct of the waste treatment. The organic waste present in the effluent is converted into microbial biomass, which is known as single-cell proteins.

18.3 MEASUREMENT AND KINETICS OF MICROBIAL GROWTH

Growth is an orderly increase in the quantity of cellular constituents. It depends on the ability of the cell to form new protoplasm from nutrients available in the environment. A proper understanding regarding microbial growth is essential to utilize the microbial process to get maximum product output. Among the various

types of microorganisms there are basically four general patterns of cell multiplication. Bacteria mainly increase in number by binary fission, yeast multiply by budding, fungi increas the biomass by the elongation of mycellium and its branching, and in the case of virus, there is no regular pattern for multiplication, mainly because its multiplication is host dependent and grow intracellularly.

Measurement of Microbial Growth

In bacteria, multiplication takes place by simple division of a cell into two by a process called **binary fission.** The growth and division of a bacterial cell involves increase in cell mass and number of ribosomes, duplication of the bacterial chromosome, synthesis of new cell wall and plasma membrane, partitioning of the two chromosomes, septum formation, and cell division. This asexual process of reproduction is called binary fission.

During this process, there is an orderly increase in cellular structures and components, replication and segregation of the bacterial DNA, and formation of a septum or cross wall, which divides the cell into two progeny cells. The process is coordinated by the bacterial membrane, perhaps by the mesosomes. The DNA molecule is believed to be attached to a point on the membrane where it is replicated. The two DNA molecules remain attached at points side-by-side on the membrane while new membrane material is synthesized between the two points. This draws the DNA molecules in opposite directions while new cell wall and membrane are laid down as a septum between the two chromosomal compartments. When septum formation is complete, the cell splits into two progeny cells. The time interval required for a bacterial cell to divide or for a population of bacterial cells to double is called the **generation time.** Generation time for bacterial species growing in nature may be as short as 15 minutes or as long as several days. For unicellular organisms such as bacteria, growth can be measured in terms of two different parameters: changes in **cell mass** and changes in **cell numbers**.

Growth Kinetics and Specific Growth Rate

When bacteria are grown in a closed system (also called a batch culture) such as a test tube, the population of cells almost always exhibits these growth dynamics: cells initially adjust to the medium (lag phase) until they can start dividing regularly in the exponential phase. When their growth becomes limited, the cells stop dividing (stationary phase), until eventually they show loss of viability (death phase). Growth is expressed as change in the number of viable cells versus time or the cell biomass versus time.

Growth of cells in a microbial culture under a steady state or balanced growth can be compared to a chemical reaction, in which the substrate is getting converted

into products. In a microbial culture under a steady state the substrate is the nutrients and the product is the cell biomass. In such a reaction the rate of growth will be proportional to the cell biomass present in the culture. The cell culture behaves like an autocatalytic reaction. When the microbial culture is under a steady state the rate of increase in cell biomass dX/dt is equal to the product of specific growth and cell concentration (biomass concentration).

$$DX/dt = \mu X \tag{1}$$

where X is the cell concentration (gm/L) and μ is the specific growth rate (in hour^{-1}).

The specific growth rate $\mu = dX/dt \times 1/X$, which is an index of rate of growth of cells in those particular conditions. Specific growth rate can be determined by plotting dX/dt against X, the cell concentration, and determining the slope of the straight line. It is possible to calculate the generation time or the doubling time of the organism or bacterial cell, if we know the initial and final cell concentration of the culture and the specific growth rate.

The cell biomass at the starting of exponential growth is X and after time, t, it is 2X. Time required for doubling the biomass or the generation time of the cell can be calculated by following the above equation (Equation No. 1).

$$\ln 2X/t = \mu X$$

$$\ln 2X/X = \mu t$$

$$\ln 2 = \mu t$$

Therefore,

$$t = \ln 2/\mu$$

$$t = 0.693/\mu \tag{2}$$

Here t is the generation time or the doubling time of the cells. If the specific growth 'μ' of the cells is calculated it can be substituted in the above equation to determine the generation time or the doubling time. From this it is very clear that the doubling time and specific growth rate are inversely related. As the doubling time increases, the specific growth rate decreases. The microbial cell cultures usually have a high specific growth rate, because they have short doubling time or generation time.

The cell numbers of the microbial culture at different time intervals can be counted and this data can be used for calculating the specific growth rate as follows:

$$\ln 2X/X = \mu t \text{ or } \ln 2X - X = \mu (t - t_0)$$

on converting natural logarithm to logarithm to the base 10

$$\log_{10} 2X - \log_{10} X = \mu/2.303 (t - t_0)$$

2X and X represent the amount of microbial cells at time t and t_0.