MASS COLLEGE OF ARTS AND SCIENCE

Kumbakonam – 612 501

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UNIT I

FERMENTATION TECHNOLOGY

MEANING OF FERMENTATION TECHNOLOGY:

Fermentation is the process involving the biochemical activity of organisms, during their growth, development, reproduction, even senescence and death. Fermentation technology is the use of organisms to produce food, pharmaceuticals and alcoholic beverages on a large scale industrial basis.

The basic principle involved in the industrial fermentation technology is that organisms are grown under suitable conditions, by providing raw materials meeting all the necessary requirements such as carbon, nitrogen, salts, trace elements and vitamins.

The end products formed as a result of their metabolism during their life span are released into the media, which are extracted for use by human being and that have a high commercial value. The major products of fermentation technology produced economically on a large scale industrial basis are wine, beer, cider, vinegar, ethanol, cheese, hormones, antibiotics, complete proteins, enzymes and other useful products.

Fermentation Methodology:

Fermentation process is carried out in a container called the fermentor or bioreactor. The design and nature of the fermentor varies depending upon the type of fermentation carried out. Invariably all the fermentors have facilities to measure some of the fermentation parameters like temperature, pressure, pH, elapsed fermentation time, liquid level, mass etc.

The different types of fermentors are:

(1) External recycle airlift fermentor—for producing bacterial biomass, with methanol as substrate.

(2) Internal recycle airlift fermentor—for producing yeast with oil as substrate.

(3) Tubular tower fermentor—Used for making beer, wine, vinegar etc.

(4) Nathan fermentor—used in brewing industry.

(5) Stirred fermentor—used for making antibiotics.

Types of Fermentation Processes:

There are three different process of fermentation viz.:

(1) Batch fermentation

(2) Feb-batch fermentation and

(3) Continuous culture.

Batch fermentation:

This term is attributed to that type of fermentation wherein there is change in culture medium, number of microorganisms and the amount of the product produced (i.e. the metabolite or target protein). In batch fermentation six phases of the microbial growth are seen.

(a) Lag phase:

Immediately after inoculation, there is no increase in the numbers of the microbial cells for some time and this period is called lag phase. This is in order that the organisms adjust to the new environment they are inoculated into.

(b) Acceleration phase:

The period when the cells just start increasing in numbers is known as acceleration phase.

(c) Log phase:

This is the time period when the cell numbers steadily increase.

(d) Deceleration phase:

The duration when the steady growth declines.

(e) Stationary phase:

The period where there is no change in the microbial cell number is the stationary phase. This phase is attained due to depletion of carbon source or accumulation of the end products.

(f) Death phase:

The period in which the cell numbers decrease steadily is the death phase. This is due to death of the cells because of cessation of metabolic activity and depletion of energy re-sources. Depending upon the product required the different phases of the cell growth are

maintained. For microbial mass the log phase is preferred. For production of secondary metabolites i.e. antibiotics, the stationary phase is preferred.

Feb-batch fermentation:

In this type of fermentation, freshly prepared culture media is added at regular intervals without removing the culture fluid. This increases the volume of the fermentation culture. This type of fermentation is used for production of proteins from recombinant microorganisms.

Continuous fermentation:

In this type of fermentation the products are removed continuously along with the cells and the same is replenished with the cell girth and addition of fresh culture media. This results in a steady or constant volume of the contents of the fermentor. This type of fermentation is used for the production of single cell protein (S.S.P), antibiotics and organic solvents.

Procedure of Fermentation:

(a) Depending upon the type of product required, a particular bioreactor is selected.

(b) A suitable substrate in liquid media is added at a specific temperature, pH and then diluted.

(c) The organism (microbe, animal/plant cell, sub-cellular organelle or enzyme) is added to it.

(d) Then it is incubated at a specific temperature for the specified time.

(e) The incubation may either be aerobic or anaerobic.

i. Aerobic conditions are created by bubbling oxygen through the medium.

ii. Anaerobic conditions are created by using closed vessels, wherein oxygen cannot diffuse into the media and the oxygen present just above is replaced by carbon dioxide released.

(f) After the specified time interval, the products are removed, as some of the products are toxic to the growing cell or at least inhibitory to their growth. The organisms are re-circulated. The process of removal of the products is called downstream processing.

Example of batch fermentation technology using tubular tower fermentor:

The simplest and the most commonly used fermentation technology is the preparation of curd from milk.

The details of the technology are as under:

The fermentor or bioreactor:

Mud pot or steel cooking vessel or a dish or a cup

The substrate:

Milk

The specific temperature:

37°C

The organism:

Preformed curd (containing the microbe Lactobacillus cecai)

The incubation:

At 37° C for 6-8 hours

The aeration:

The process is anaerobic

The process:

Preformed curd has microbes that utilize lactose present in the milk. Lactose is hydrolysed into glucose and galactose. Galactose is converted to glucose. Glucose is broken down to lactic acid by glycolytic pathway. The lactic acid produced, lowers the pH of milk from 6.6 to 4.5. The isoelectric pH of milk protein-casein is 4.5. At this pH, casein precipitates forming fine micelles in the milk thereby curdling it.

Milk is warmed before adding it to the fermentor so as to maintain the temperature required for the growth of the microbes. The container/fermentor is not disturbed (no stirring is taken up) so that the precipitation is uniform.

The elapsed time i.e. the time required to form the curd is crucial; it depends upon the atmospheric temperature. During summer the curd is formed within 4-6 hours, whereas during rainy season it takes 6-8 hours and in winter it takes almost 8-12 hours. The process is anaerobic hence it is better to keep the vessel closed. Though it cannot be air tight but still the surface of the milk that contains fat prevents air from penetrating in the liquid, furthermore the metabolizing microbes replace the oxygen with carbon dioxide released.

Example of continuous fermentation technology using stirred fermentor:

Another common fermentation technology is the process of food digestion.

The details of the technology are as under:

The fermentor or bioreactor:

Stomach (G.I. tract)

The substrate:

Food

The specific temperature:

37°C

The organism:

Enzymes, microbes, acids etc.

The Incubation:

At 37°C for 3-4 hours

The aeration:

The process is anaerobic

The process:

The food eaten by us is stored in the stomach where HC1 and some enzymes are secreted that convert food into chyme (semi solid). The food stays for 3-4 hours at 37° C. The digested

food is absorbed in the blood and the undigested food is excreted. This is a continuous fermentation because the substrate (food) is continuously added and the products (digested/undigested material) are continuously removed. Stomach-the fermentor is a stirred type i.e. the peristaltic movement of the gastro-intestinal tract mixes the food.

Categories of Fermentation Technology:

Fermentation technology can be grouped into four major categories viz.

1. Microbial biomass production:

Microbial cells (biomass) are grown commercially as continuous culture on a large scale (1500/m^3). The microbial cells including algae, bacteria, yeasts, moulds and mushrooms are dried and used as a good source of a complete protein called 'single cell protein (SCP)' which serves as human food or animal feed. The incubation of wheat flour for preparation of 'tandoori roti' and rice flour for 'dosa' are good examples for the production of single cell protein.

These two food stuffs or the common food are usually lacking or not having sufficient quantities of lysine and methionine amino acids. Single cell protein produced by different sources is a rich source of different essential amino acids, thereby supplementing the amino acid lacking in a particular food. The incubation of the foods results in the growth of the microbial biomass, producing the SCP.

SCP produced by some microorganisms is lysine rich whereas those produced by others are methionine rich. The substrates used by the microbes producing the single cell protein range from carbohydrates to hydrocarbons and petrochemicals. Other organisms use the gases—methane, ethane, propane, n-butane etc as substrate for fermentation.

SCP producing organisms and their substrates

Member	Substrate used
Chlorella pyrenoidesa	CO_2 (0.7% in air), fluorescent light
Scenedesmus acutus	CO_2, sunlight
Spirulina maxima	CO_2 (0.5%), $NaHCO_3$, sunlight
Achromobacter deluacuate	Diesel oil
Nocardia sps.	n-Alkanes
Pseudomonas sps.	Fuel oil
Candida tropicalis	Molasses
Trichoderma sps.	Coffee wastes

Lysine-rich SCP (7g lysine/16 g N) producing organisms are Chlorella sorokiniana, Cellulomonas alkaligens, Saccharomyces cerevisiae etc. Methionine-rich SCP (2g methionine/16 g N) producing organisms are Methylococcus capsulatus, Saccharomyces cerevisiae and Aspergillus.

2. Microbial metabolites:

During the metabolism of microbial cells a number of compounds are produced and many are secreted out of the cell, which can be easily extracted and are very useful to man and animals. Therefore fermentation by microbial cells is carried out on an industrial scale, in order to get various metabolites.

The metabolites produced by the microbes can be grouped into two categories:

(a) Primary metabolites and

(b) Secondary metabolites.

(a) Primary metabolites:

Metabolites which are produced by the metabolism required for the maintenance of the minimum life process of a microbe are known as primary metabolites. The primary metabolites are produced in abundance at an early stage of growth. Examples of primary metabolites are ethanol, citric, acid, glutamic acid, lactic acid, acetic acid, acetone, formic acid, butanol, propionic acid, dihydroxy-acetone, glycerol etc. These metabolites are produced by fermentation technology applying different microbes under varying conditions of fermentation.

(b) Secondary metabolites:

Secondary metabolites are those metabolites, which are not produced directly by the metabolism required for the vital life process of microbes, instead are produced by some specialized metabolic process. However most of the secondary metabolites are derived from the primary metabolites. The secondary metabolites include the antibiotics, alkaloids, toxic pigments, vitamins etc.

Antibiotics:

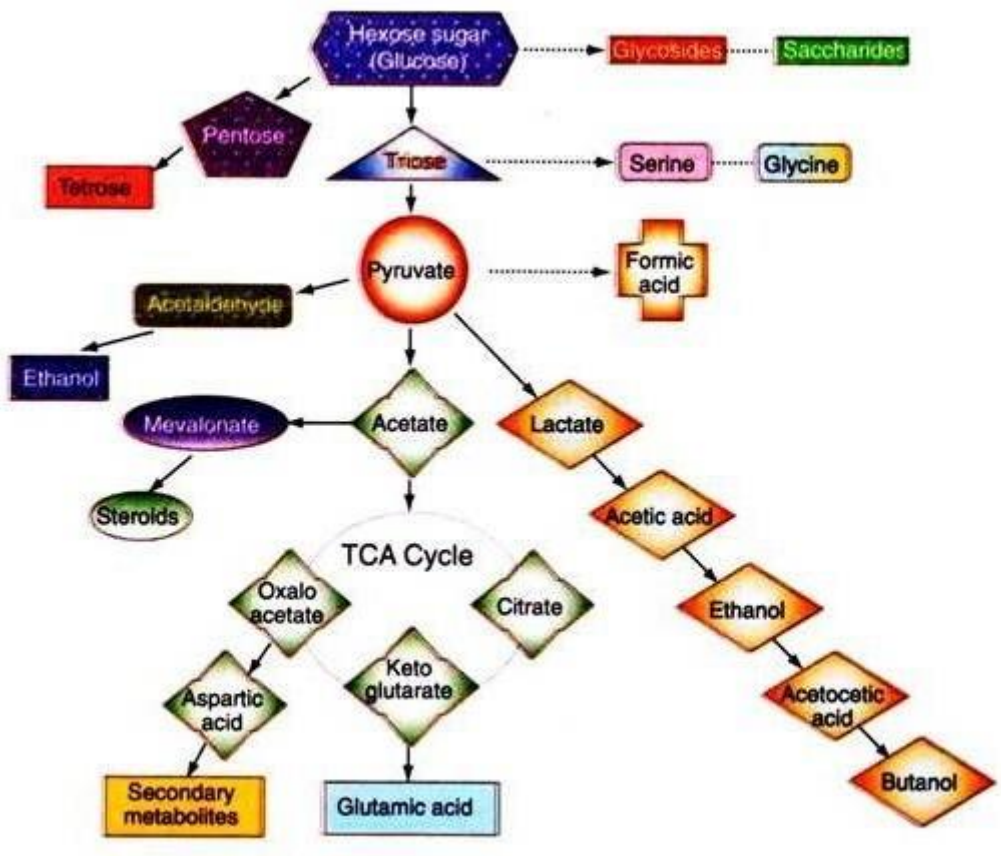
An antibiotic is a substance produced by a microorganism which can inhibit growth or completely destroy other microorganisms. The antibiotics are not synthesized by the microorganism by a single gene, instead a set of 10-20 genes takes part in the synthesis of an antibiotic. The genes for antibiotic are mostly located on the plasmid, however in some microbes they are found on the chromosomal DNA.

About 45000 different antibiotics have been discovered so far, but only about 100 are used for human treatment, because only those antibiotics can be used which are toxic to the invading microbe but non-toxic for the human.

Some of the antibiotics and the microbes which produce them are:

Amphotericin-B—Streptomyces nodosus Chloramphenicol—Streptomyces venezuelae, Erythromycin—S. eythirus, Gentamycin—Micromonospora purpurea, Gramicidin—Bacillus brevis, Penicillin—Penicillin chryosogen Streptomycin—S. grises, Tetracycline—S. aureofacins.

These microbes are grown under suitable fermentation conditions so as to get the desired antibiotic. Mutant microbes are used for industrial production of antibiotics.



Pathways showing production of primary metabolites

3. Microbial enzymes:

When microbes are cultured, they secrete some enzymes into the media and these enzymes are extracted and widely used in several industries like detergent, food processing, brewing and pharmaceutical. They are also used for diagnostic, scientific and analytical purposes. Biotechnological methods are used to engineer microbial cells so as to induce them to

produce enzymes like renin by E. coli and amylases by Bacillus stearothermophillis.

These enzymes are generally bound to matrix or in some manner retained in the reactor to be reused and hence these are called immobilized enzymes. In some cases the microorganism producing enzyme is immobilized. Some of the enzymes produced by fermenting microbes are—Glucose oxidase, protease glucoamylase, amylase, glucose isomerase, rennin, pectinase, superoxide dismutase, cellulase, invertase, lactase and lipase.

Some thermophile bacteria produce enzymes that are thermo-stable and which can be used in industrial processes at high temperatures, ex. glyceraldehyde-3-phosphate dehydrogenase, phosphofructo-kinase, alcohol dehydrogenase, superoxide dismutase and restriction

endonucleases, which are produced by Bacillus stearothermophillis thermoysia. Further, genes for thermophilic enzymes are introduced into E. coli, which is cultured for producing the thermophilic enzymes.

4. Bioconversion, biotransformation or modification of the substrate:

The fermenting microbes have got the capacity to convert an added substrate into some more valuable product, ex. conversion of ethanol to acetic acid (vinegar), isopropanol to acetone, glucose to gluconic acid, sorbitol to sorbose (this is used in the manufacture of vitamin C), sterols to steroids.

Among all these bioconversions, the production of steroids is the most widely applied fermentation biotechnology for the conversion of sterols into steroids, like cortisone, hydroxycortisone, prednisone, dexamethasone, testosterone, estradiol etc. Hitherto, steroids were produced by chemical methods, which were laborious and costly.

For instance the chemical synthesis of cortisone required 37 steps under extreme conditions. One of the steps is introduction of oxygen at position 11 in the steroid nucleus. The microbe Rhizopus arrhizus being capable of hydroxylating progesterone (a steroid) at position 11 is used in the fermentation to produce progesterone an intermediate in the synthesis of cortisone. Thus, this reduced the chemical synthesis steps from 37 to 11 and all under normal conditions, thereby making it economical and easy. Steroids are used as anti-inflammatory agents, contraceptives, treating hormonal insufficiency, allergy, skin diseases etc.

Downstream processing (DSP):

The method by which the products of fermentation are recovered and separated is known as downstream processing. This forms the major (about 85%) portion of the complete fermentation technology. There are various methods by which DSP is carried out.

First of all, the broth is conditioned i.e. the cells are aggregated and form large clumps, which makes the separation easier. The conditioning is done by heating, freezing, pH change, antigen-antibody reactions etc. Then the conditioned broth is used for the separation of the constituents for which techniques like sedimentation, floatation, filtration, ultra-filtration, centrifugation and micro-filtration are applied.

Fermentation

Fermentation Definition

Fermentation refers to the metabolic process by which organic molecules (normally glucose) are converted into acids, gases, or alcohol in the absence of oxygen or any electron transport chain. Fermentation pathways regenerate the coenzyme nicotinamide adenine dinucleotide (NAD^+), which is used in glycolysis to release energy in the form of adenosine triphosphate (ATP). Fermentation only yields a net of 2 ATP per glucose molecule (through glycolysis), while aerobic respiration yields as many as 32 molecules of ATP per glucose molecule with the aid of the electron transport chain.

The study of fermentation and its practical uses is named zymology and originated in 1856 when French chemist Louis Pasteur demonstrated that fermentation was caused by yeast. Fermentation occurs in certain types of bacteria and fungi that require an oxygen-free environment to live (known as obligate anaerobes), in facultative anaerobes such as yeast, and also in muscle cells when oxygen is in short supply (as in strenuous exercise). The processes of fermentation are valuable to the food and beverage industries, with the conversion of sugars into ethanol used to produce alcoholic beverages, the release of CO_2 by yeast used in the leavening of bread, and with the production of organic acids to preserve and flavor vegetables and dairy products.

Function of Fermentation

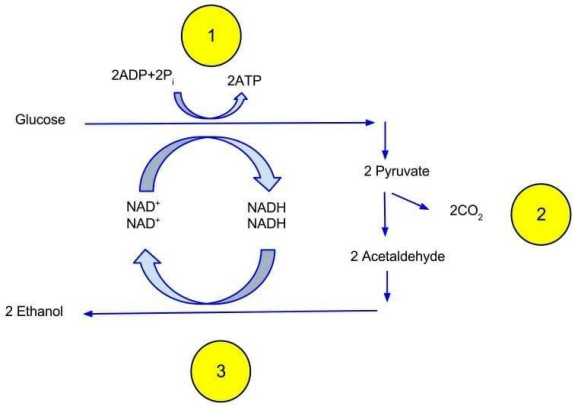
The main function of fermentation is to convert NADH back into the coenzyme NAD^+ so that it can be used again for glycolysis. During fermentation, an organic electron acceptor (such as pyruvate or acetaldehyde) reacts with NADH to form NAD^+, generating products such as carbon dioxide and ethanol (ethanol fermentation) or lactate (lactic acid fermentation) in the process.

Types of Fermentation

There are many types of fermentation that are distinguished by the end products formed from

pyruvate or its derivatives. The two fermentations most commonly used by humans to produce commercial foods are ethanol fermentation (used in beer and bread) and lactic acid fermentation (used to flavor and preserve dairy and vegetables).

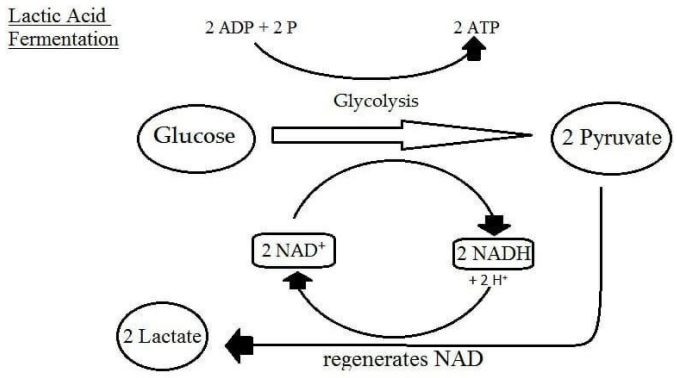
Ethanol Fermentation



In ethanol fermentation, the pyruvate produced through glycolysis is converted to ethanol and carbon dioxide in two steps. First, the pyruvate releases carbon dioxide to form a two-carbon compound called acetaldehyde. Next, acetaldehyde is reduced by NADH to ethanol, thereby regenerating the NAD^+ for use in glycolysis. Overall, one molecule of glucose is converted into two molecules of carbon dioxide and two molecules of ethanol.

Ethanol fermentation is typically performed by yeast, which is a unicellular fungus.

Lactic Acid Fermentation



This figure depicts the processes of glycolysis and homolactic fermentation.

There are two main types of lactic acid fermentation: homolactic and heterolactic. In homolactic acid fermentation, NADH reduces pyruvate directly to form lactate. This process does not release gas. Overall, one molecule of glucose is converted into two molecules of

lactate. In heterolactic fermentation, some lactate is further metabolized, resulting in ethanol and carbon dioxide via the phosphoketolase pathway.

Lactic acid fermentation is primarily performed by certain types of bacteria and fungi. However, this type of fermentation also occurs in muscle cells to produce ATP when the oxygen supply has been depleted during strenuous exercise and aerobic respiration is not possible.

Fermentation Equation

Ethanol Fermentation

GLUCOSE → 2 ETHANOL + 2 CARBON DIOXIDE

$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$

Lactic Acid Fermentation

GLUCOSE → 2 LACTIC ACID

$C_6H_{12}O_6 \rightarrow 2\ C_3H_6O_3$

Products of Fermentation

While there are a number of products from fermentation, the most common are ethanol, lactic acid, carbon dioxide, and hydrogen gas (H_2). These products are used commercially in foods, vitamins, pharmaceuticals, or as industrial chemicals. In addition, many less common products still offer commercial value. For example, the production of acetone via the acetone – butanol – ethanol fermentation was first developed by the Jewish chemist Chaim Weizmann and was important to the British war industry during Word War I.

TYPES OF FERMENTATION- SUBMERGED, SOLID AND SURFACE TYPE

Introduction

Fermentation is one of the oldest technologies used for food preservation. Over the centuries, it has evolved, been refined and diversified. Today a variety of fermented foods is produced both in industrialised and developing countries using this technology at the s. A wide range of raw materials is used as substrates and panoply of products is concocted. Foods derived from fermentation are major constituents of the human diet all over the world. Although advances in food science and technology have given rise to a wide range of new food technologies, fermentation has remained an important technology throughout the history of mankind. Many benefits are attributed to fermentation. It preserves and enriches food, improves digestibility, and enhances the taste and flavour of foods. It is also an affordable technology and is thus accessible to all populations. Furthermore, fermentation has the potential of enhancing food

safety by controlling the growth and multiplication of a number of pathogens in foods. Thus, it makes an important contribution to human nutrition, particularly in developing countries, where economic problems pose a major barrier to ensuring food safety.

Fermentation systems may be **liquid**, also known as **submerged** or **solid state**, also known as **surface**. Most fermentors used in industry are of the submerged type, because the submerged fermentor saves space and is more amenable to engineering control and design.

Submerged Liquid Fermentations

Submerged liquid fermentations are traditionally used for the production of microbially derived enzymes. Submerged fermentation involves submersion of the microorganism in an aqueous solution containing all the nutrients needed for growth.

Fermentation takes place in large vessels (fermenter) with volumes of up to 1,000 cubic metres. The fermentation media sterilises nutrients based on renewable raw materials like maize, sugars and soya. Most industrial enzymes are secreted by microorganisms into the fermentation medium in order to break down the carbon and nitrogen sources. Batch-fed and continuous fermentation processes are common. In the batch-fed process, sterilised nutrients are added to the fermenter during the growth of the biomass. In the continuous process, sterilised liquid nutrients are fed into the fermenter at the same flow rate as the fermentation broth leaving the system. Parameters like temperature, pH, oxygen consumption and carbon dioxide formation are measured and controlled to optimize the fermentation process.

Next in harvesting enzymes from the fermentation medium one must remove insoluble products, e.g. microbial cells. This is normally done by centrifugation. As most industrial enzymes are extracellular (secreted by cells into the external environment), they remain in the fermented broth after the biomass has been removed. The enzymes in the remaining broth are then concentrated by evaporation, membrane filtration or crystallization depending on their intended application. If pure enzyme preparations are required, they are usually isolated by gel or ion exchange chromatography.

Several types of submerged type of fermentors are known and they may be grouped in several ways: shape or configuration, whether aerated or anaerobic and whether they are batch or continuous. The most commonly used type of fermentor is the Aerated Stirred Tank Batch Fermentor.

Aerated stirred tank batch fermentor

A *typical* fermentor of this type (as shown in Fig 22.1 and Fig. 22.2) is an upright closed cylindrical tank fitted with one or more baffles attached to the side of the wall, a water jacket or coil for heating and/ or cooling, a device for forcible aeration (known as sparger), a mechanical agitator usually carrying a pair or more impellers, means of introducing organisms and nutrients and of taking samples, and outlets for exhaust gases. Modern fermentors are highly

automated and usually have means of continuously monitoring, controlling or recording pH, oxidation-reduction potential, dissolved oxygen, effluent O2 and CO2, and chemical components. Further diagrams of stirred tank fermentors are shown below

Solid-State Fermentation (SSF)

The origin of Solid-state fermentation can be traced back to bread-making in ancient Egypt. Solid substrate fermentations also include a number of well known microbial processes such as soil growth, composting, silage production, wood rotting and mushroom cultivation. In addition, many familiar western foods such as mold-ripened cheese, bread, sausage and many foods of Asian origin including miso, tempeh and soy sauce are produced using SSF. Beverages derived from SSF processes include ontjom in Indonesia, shao-hsing wine and kaoliang (sorghum) liquor in China and sake in Japan.

SSF is used for the production of bioproducts from microorganisms under conditions of low moisture content for growth. The medium used for SSF is usually a solid substrate (e.g., rice bran, wheat bran, or grain), which requires no processing. In order to optimize water activity requirements, which are of major importance for growth, it is necessary to take into account the water sorption properties of the solid substrate during the fermentation. In view of the low water content, fewer problems due to contamination are observed. The power requirements are lower than submerged fermentation. Inadequate mixing, limitations of nutrient diffusion, metabolic heat accumulation, and ineffective process control renders SSF generally applicable for low value products with less monitoring and control. There exists a potential for conducting SSF on inert substrate supports impregnated with defined media for the production of high value products.

It involves the growth of microorganisms on moist solid particles, in situations in which the spaces between the particles contain a continuous gas phase and a minimum of visible water.

Although droplets of water may be present between the particles, and there may be thin films of water at the particle surface, the inter-particle water phase is discontinuous and most of the inter- particle space is filled by the gas phase. The majority of the water in the system is absorbed within the moist solid-particles the more general term "solid-substrate fermentation" is used to denote any type of fermentation process that involves solids, including suspensions of solid particles in a continuous liquid phase and even trickling filters.

Advantages of Solid State Fermentation over Submerged Fermentation

1. Higher volumetric productivity
2. Usually simpler with lower energy requirements
3. Might be easier to meet aeration requirements
4. Resembles the natural habitat of some fungi and bacteria
5. Easier downstream processing
6. The fungal hyphae are bathed in a liquid medium and do not run the risk of desiccation;
7. Temperature control is typically not overly difficult, such that the organism is exposed to a constant temperature throughout its growth cycle;
8. The availability of O_2 to the biomass can be controlled reasonably well at a particular level of saturation of the medium
9. The availability of the nutrients to the organism can be controlled within relatively narrow limits if desired, through the feeding of nutrient solutions.
10. Although shear forces do occur within mechanically stirred bioreactors, the nature and magnitude of these forces are well understood and it is possible to use bioreactors that provide a low-shear environment, if the organism is highly susceptible to shear damage, such as bubble columns or air lift bioreactors;
11. pH control is relatively easy to provide.

In contrast, the environment in SSF can be quite stressful to the organism. For example:

1. Fungal hyphae are exposed to an air phase that can desiccate them;
2. Temperatures can rise to values that are well above the optimum for growth due to the inadequate removal of waste metabolic heat. In other words, the temperature to which the organism is exposed can vary during the growth cycle;

3. O_2 is typically freely available at the surface of the particle, however, there may be severe restrictions in the supply of O_2 to a significant proportion of the biomass that is within a biofilm at the surface or penetrating into the particle;
4. The availability of nutrients to the organism may be poor, even when the average nutrient concentration within the substrate particle, determined after homogenizing a sample of fermenting solid particles, is high. In other words, there tend to be large concentration gradients of nutrients within the particles; movement of the particles of the solid substrate can cause impact and shear damage. In the case of fungal processes the hyphae can suffer severe damage
5. It may be difficult to provide pH control under some circumstances.

However, there are certain instances in which, despite being more problematic, SSF may be appropriate:

1. When the product needs to be in a solid form (e.g., fermented foods);
2. When a particular product is only produced under the conditions of SSF or, if produced in both SLF and SSF, is produced in much higher levels in SSF. For example, certain enzymes are only induced in SSF and some fungi only sporulate when grown in SSF, in which the hyphae are exposed directly to an air phase. For example, *Monascus* pigment and many fungal spores are produced in much higher yields in SSF
3. If it is desired to use genetically unmodified organisms in a process for the production of such a product, then SSF may be the only option;
4. When socio-economic conditions mean that the fermentation process must be carried out by relatively unskilled workers. Some SSF processes can be relatively resistant to being overtaken by contaminants;
5. When the product is produced in both SSF and SLF, but the product produced in SSF has desirable properties which the product produced in SLF lacks. For example, spore-based fungal biopesticides produced in SSF processes are usually more resistant to adverse conditions than those produced in SLF, and are therefore more effective when spread in the field;
6. When it is imperative to use a solid waste in order to avoid the environmental impacts that would be caused by its direct disposal. This is likely to become an increasingly

important consideration as the ever-increasing population puts an increasing strain on the environment.

Some examples of traditional SSF processes are:

1. Tempe, which involves the cultivation of the fungus *Rhizopus oligosporus* on cooked soybeans.
2. The *koji* step of soy sauce manufacture, which involves the cultivation of the fungus *Aspergillus oryzae* on cooked soybeans.
3. 'ang-kak', or "red rice", which involves the cultivation of the fungus *Monascus purpureus* on cooked rice.

Beyond this, over the last three decades, there has been an upsurge in interest in SSF technology, with research being undertaken into the production of a myriad of different products, including:

1. Enzymes such as amylases, proteases, lipases, pectinases, tannases, cellulases, and rennet;
2. Pigments;
3. Aromas and flavor compounds;
4. "Small organics" such as ethanol, oxalic acid, citric acid, and lactic acid;
5. Gibbrellic acid (a plant growth hormone);
6. Protein-enriched agricultural residues for use as animal feeds;
7. Animal feeds with reduced levels of toxins or with improved digestibility;
8. Antibiotics, such as penicillin and oxytetracycline;
9. Biological control agents, including bioinsecticides and bioherbicides;
10. Spore inocula (such as spore inoculum of *Penicillium roqueforti* for blue cheese production).
11. Decolorization of dyes;
12. Biobleaching;
13. Biopulping;
14. Bioremediation.

Bacteria, yeast and fungi can all grow on solid substrates and have applications in SSF processes. However, filamentous fungi are the best adapted species for SSF and dominate in the research and practical applications around the world. Bacterial SSF fermentations are

rarely used for large scale enzyme production, but are very important in nature and in the fermented food industry. Filamentous fungi are the most important group of microorganisms for enzyme production in SSF. The hyphal mode of growth gives a major advantage to filamentous fungi over unicellular microorganisms in the colonization of solid substrates and the utilization of available nutrients. The filamentous fungi have the power to penetrate solid substrates. Hydrolytic enzymes are excreted at the hyphal tip, without large dilution. This makes the action of hydrolytic enzymes very efficient and allows penetration into most solid substrates. This is critical for the growth of the fungi. Fungi cannot transport macromolecular substrates across the cell wall, so the macromolecule must be hydrolyzed externally into soluble units that can be transported into the cell.

Fundamentally, there are 6 types of solid-state fermenters:

1. Tray bioreactor
2. Packed bed bioreactor
3. Rotary drum bioreactor
4. Swing solid state bioreactor
5. Stirred vessel bioreactor
6. Air solid fluidized bed bioreactor

The simplest SSF reactor is the tray. In a tray bioreactor a relatively thin layer of substrate is spread over a large horizontal area. There is no forced aeration, although the base of the tray may be perforated and air forced around the tray. Mixing, if any, is by simple automatic devices or manual. Internal temperature may vary with ambient temperature; or the tray may be placed in a temperature-controlled room. Tray bioreactors have been used successfully at laboratory, pilot, semi-commercial and commercial scale.

UNIT II

PRODUCTION OF IMPORTANT ORGANIC ACIDS BY FERMENTATION ARE GIVEN BELOW:

1. Citric Acid:

Citric acid was first discovered as a constituent of lemon. Today, we know citric acid as an intermediate of ubiquitous Krebs cycle (citric acid cycle), and therefore, it is present in every living organism. In the early days, citric acid was isolated from lemons (that contain 7-9% citric acid), and today about 99% of the world's citric acid comes from microbial fermentation.

Applications of Citric Acid:

1. Citric acid, due to its pleasant taste and palatability, is used as a flavoring agent in foods and beverages e.g., jams, jellies, candies, desserts, frozen fruits, soft drinks, wine. Besides brightening the colour, citric acid acts as an antioxidant and preserves the flavors of foods.

2. It is used in the chemical industry as an antifoam agent, and for the treatment of textiles. In metal industry, pure metals are complexed with citrate and produced as metal citrates.

3. In pharmaceutical industry, as trisodium citrate, it is used as a blood preservative. Citric acid is also used for preservation of ointments and cosmetic preparations. As iron citrate, it serve as a good source of iron.

4. Citric acid can be utilized as an agent for stabilization of fats, oils or ascorbic acid. It forms a complex with metal ions (iron, copper) and prevents metal catalysed reactions. Citric acid is also used as a stabilizer of emulsions in the preparation of cheese.

5. In detergent/cleaning industry, citric acid has slowly replaced polyphosphates.

Microbial Strains for Citric Acid Production:

Many microorganisms can produce citric acid. The fungus Aspergillus Niger is most commonly used for industrial production of citric acid. The other organisms (although less important) include A. clavatus, A. wentii, Penicillium luteum, Candida catenula, C. guilliermondii and Corynebacterium sp.

For improved industrial production of citric acid, mutant strains of A. Niger have been developed. The strains that can tolerate high sugar concentration and low pH with reduced synthesis of undesirable byproducts (oxalic acid, isocitric acid and gluconic acid) are industrially important.

Microbial Biosynthesis of Citric Acid:

Citric acid is a primary metabolic product (of primary metabolism) formed in the tricarboxylic acid (Krebs) cycle. Glucose is the predominant carbon source for citric acid production. The biosynthetic pathway for citric acid production involves glycolysis wherein glucose is converted to two molecules of pyruvate. Pyruvate in turn forms acetyl CoA and oxaloacetate which condense to finally give citrate. The major steps in the biosynthesis of citric acid are depicted in Fig. 24.1.

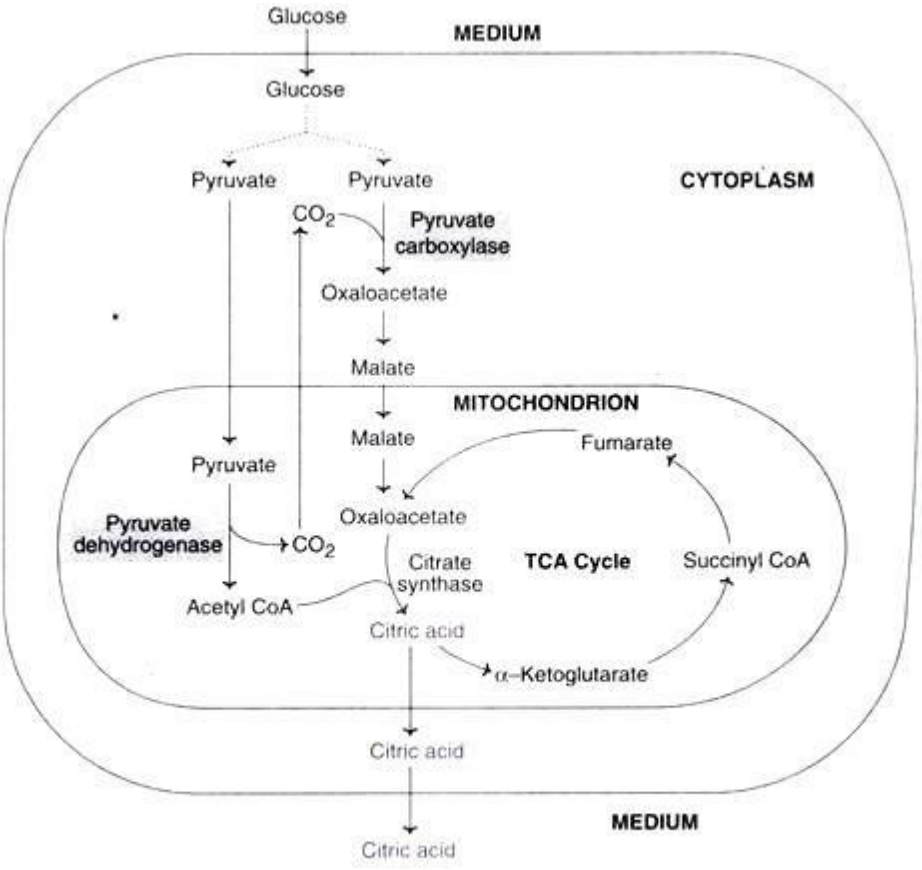


Fig. 24.1 : An outline of metabolic pathway for the biosynthesis of citric acid (TCA cycle-Tricarboxylic acid cycle).

Enzymatic regulation of citric acid production:

During the synthesis of citric acid, there is a tenfold increase in the activity of the enzyme citrate synthase while the activities of other enzymes (aconitase, isocitrate dehydrogenase) that degrade citric acid are reduced. However, recent evidence does not support the theory that reduction in the operation of tricarboxylic acid (i.e. degradation of citric acid) contributes to accumulation of citric acid.

Increased citric acid is more likely due to enhanced biosynthesis rather than inhibited degradation. Further, there are anaplerotic reactions that replenish the TCA cycle intermediates to keep the cycle continuously in operation. Pyruvate carboxylase that converts pyruvate to oxaloacetate is also a key enzyme in citric acid production.

Yield of citric acid:

Theoretically, the yield of citric acid for the most commonly used substrate sucrose has been calculated. It is worked out that from 100 g sucrose, 112 g of anhydrous citric acid or 123 g of citric acid — 1 hydrate can be formed. However, due to oxidation of sugar to CO_2 during trophophase, the yield of citric acid is lower than the calculated.

Factors in the Regulation of Citric Acid Production:

Strict maintenance of controlled nutrient conditions is very crucial for maximal production of citric acid. The optimal conditions that have been worked out for A. Niger for the production of citric acid are briefly described (Table 24.1).

TABLE 24.1 Optimal parameters/conditions for citric acid production

Condition/parameter	*Optimum*
Sugar concentration	10–25%
Trace metal concentration	
Manganese	$<10^{-8}$ M
Zinc	$<10^{-7}$ M
Iron	$<10^{-4}$ M
pH	1.5–2.5
Dissolved O_2 tension	150 mbr
Ammonium salts concentration	>0.2%
Time	150–250 hours

Carbohydrate source:

A wide range of raw materials can be used for the supply of carbohydrates. These include molasses (sugar cane or sugar beet), starch (from potatoes), date syrup, cotton wastes, banana extract, sweet potato pulp, and brewery waste and pineapple waste water.

A high yield of citric acid production occurs if the sugars that are rapidly metabolised are used e.g. sucrose, glucose, maltose. At present, cane molasses and beet molasses are commonly used. The variations in the composition of molasses (seasonal and production level), have to be carefully considered for optimising citric acid production.

The concentration of carbohydrate significantly influences citric acid production. Ideally, the sugar concentration should be 12-25%. At a concentration less than 5% sucrose, citric acid formation is negligible, and increases as the concentration is raised to 10% and then stabilizes (Fig. 24.2). It is believed that a high sugar concentration induces increased glucose uptake and consequently enhanced citric acid production.

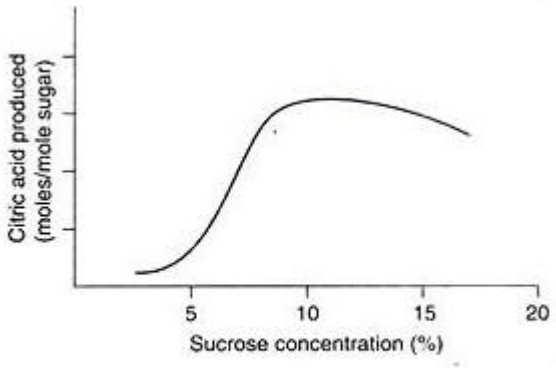


Fig. 24.2 : Effect of sugar concentration on citric acid production.

Trace metals:

Certain trace elements (Fe, Cu, Zn, Mn, Mg, Co) are essential for the growth of A. Niger. Some of the trace metals particularly Mn^{2+}, Fe^{3+} and Zn^{2+} increase the yield of citric acid. The effect of manganese ions has been investigated to some extent. These ions promote glycolysis and reduce respiration; both these processes promote citric acid production.

As regards iron, it is a cofactor for the enzyme aconitase (of TCA cycle). It is estimated that an Fe concentration of 0.05-0.5 ppm is ideal for optimal citric acid production. At higher Fe concentration, the yield is lower which can be reversed to some extent by adding copper.

pH:

The pH of the medium influences the yield of citric acid, and it is maximal when pH is below 2.5. At this pH, the production of oxalic acid and gluconic acid is suppressed. Further, at low pH, transport of citric acid is much higher. If the pH is above 4, gluconic acid accumulates at the expense of citric acid. And when the pH goes beyond 6, oxalic acid accumulates. Another advantage with low pH is that the risk of contamination is very minimal, since many organisms cannot grow at this pH.

Dissolved O_2:

The yield of citric acid production substantially increases when the dissolved O_2 tension is higher. This can be achieved by strong aeration or by sparging with pure O_2. It has been observed that sudden interruptions in O_2 supply (as occurs during power breakdowns) cause drastic reduction in citric acid production without harming the growth of the organism.

Nitrogen source:

Ammonium salts, nitrates and urea are the nitrogen sources used in the media for citric acid production. All the three compounds are equally good sources, as long as they do not adversely affect the pH of the medium. If molasses are used for nutrient supply, addition of extra nitrogen source is not required. However, some workers have shown that exogenous addition of ammonium ions stimulates citric acid production.

Production Processes for Citric Acid:

There are two processes by which citric acid can be industrially produced — the surface process and submerged process (Fig. 24.3).

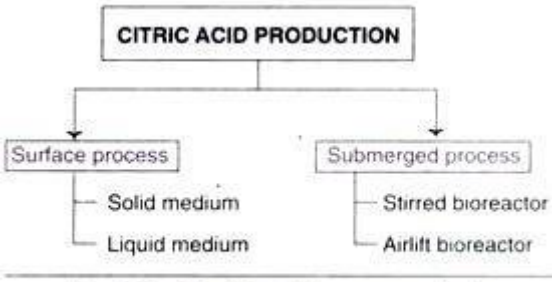


Fig. 24.3 : The industrial processes for the production of citric acid.

The surface process:

This is characterized by growing the microorganisms as a layer or a film on a surface in contact with the nutrient medium, which may be solid or liquid in nature. Thus, the surface process has supported-growth systems.

The submerged process:

In this case, the organisms are immersed in or dispersed throughout the nutrient medium. There are two types of submerged fermenters (bioreactors) stirred bioreactors and airlift bioreactors.

Surface Processes:

Solid surface fermentation:

Surface processes using solid substrates are particularly carried out in less developed areas of some Asian countries. The solid substrates such as wheat bran or pulp from sweet potato starch are used, as culture media. The pH of the medium is adjusted to 4-5, and then sterilized. Now the inoculum in the form of spores of A. niger is spread as layers (3-6 cm thickness) and incubated at 28°C.

The growth of the organisms can be accelerated by the addition of α-amylase. Solid-state fermentation takes about 80 to 100 hours for maximal production of citric acid. At the end of the process, citric acid can be extracted into hot water and isolated.

Liquid surface fermentation:

Surface fermentation using liquid as nutrient medium is the oldest method for citric acid production. It is still in use due to a simple technology, low energy costs and higher reproducibility. Further, the interference of trace metals and dissolved O_2 tension are minimal. The labour costs are however, higher since the manpower requirements are more for

cleaning the systems. About 20% of the citric acid in the world is produced by surface processes.

The nutrient supply for surface fermentation normally comes from beet molasses. The fermentation is usually carried out in aluminium trays filled with sterile nutrient medium. The inoculum in the form of spores is sprayed over the medium. A sterile air is passed for supplying O_2 as well as cooling. The temperature is maintained around 30°C during fermentation.

As the spores germinate (that occurs within 24 hours of inoculation), a layer of mycelium is formed over the medium. The pH of the nutrient medium falls to less than 2, as the mycelium grows in size and forms a thick layer on the surface of the nutrient solution. The fermentation is stopped after 7-15 days.

The mycelium and nutrient solution are separated. The mycelium is mechanically pressed and thoroughly washed to obtain maximum amount of citric acid. The nutrient solution is subjected to processing for the recovery of citric acid. The final yield of citric acid is in the range of 0.7-0.9 of per gram of sugar.

Submerged Processes:

Around 80% of the world's supply of citric acid is produced by submerged processes. This is the most preferred method due to its high efficiency and easy automation. The disadvantages of submerged fermentation are — adverse influence of trace metals and other impurities, variations in O_2 tension, and advanced control technology that requires highly trained personnel.

Two types of bioreactors are in use— stirred tanks and aerated towers. The vessels of the bioreactors are made up of high-quality stainless steel. The sparging of air occurs from the base of the fermenter.

The success and yield of citric acid production mainly depend on the structure of mycelium. The mycelium with forked and bulbous hyphae and branches which aggregate into pellets is ideal for citric acid formation. On the other hand, no citric acid production occurs if the mycelium is loose and filamentous with limited branches. An adequate supply of O_2 (20-25% of saturation value) is required for good production of citric acid. The ideal aeration rate is in the range of 0.2-1 vvm (volume/ volume/ minute).

The submerged fermenters have the problem of foam formation which may occupy about 1 /3rd of the bioreactor. Antifoam agents (e.g. lard oil) and mechanical antifoam devices are used to prevent foaming. Nutrient concentration is very important in the industrial production of citric acid. A diagrammatic representation of sucrose, citric acid and biomass concentration with respect to cultivation time is shown in Fig. 24.4. It is estimated that under optimal conditions, in about 250-280 hours, 100- 110 g/l of citric acid is obtained from 140 g/l of sucrose with a biomass (dry weight) of 8-12 g/l.

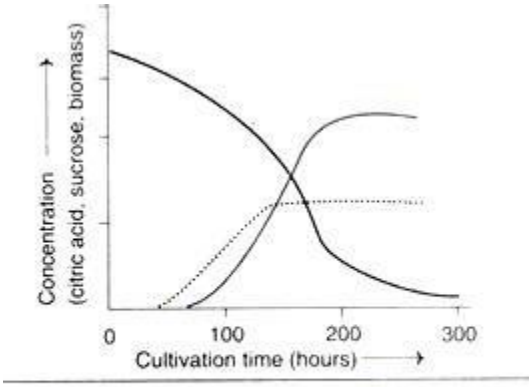


Fig. 24.4 : A diagrammatic representation of citric acid (—) production along with sucrose (—) and biomass (- - -) concentration in relation to time.

Production of Citric Acid from Alkanes:

Both yeasts and bacteria can be used for citric acid production from n-alkanes (C_9-C_{23} hydrocarbons). The citric acid yield is better from hydrocarbons compared to sugars i.e. 145% of citric acid from paraffin. The most commonly used organism is Candida lipolytica. The fermentation can be carried out in batch, semi-continuous or continuous modes. The pH should be kept above 5. The major limitations of citric acid production from alkanes are—very low solubility of alkanes and increased production of unwanted isocitric acid.

Recovery of Citric Acid:

The steps for the recovery of citric acid either from surface process or submerged process are comparable (Fig. 24.5). The recovery starts with the filtration of the culture broth and washing of mycelium (which may contain about 10% of citric acid produced). Oxalic acid is an unwanted byproduct and it can be removed by precipitation by adding lime at pH < 3.

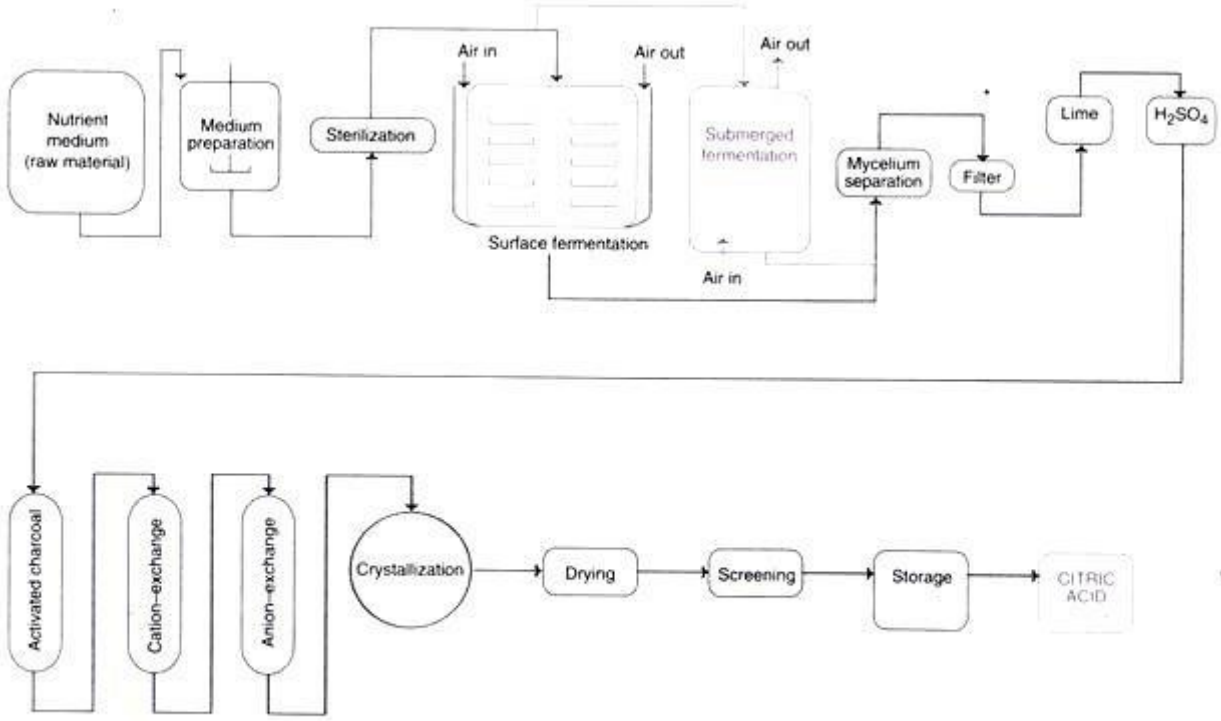


Fig. 24.5 : Flow chart for industrial production of citric acid by surface or submerged processes.

The culture broth is then subjected to pH 7.2 and temperature 70-90°C for precipitating citric acid. For further purification, citric acid is dissolved in sulfuric acid (calcium sulfate precipitate separates). The final steps for citric acid recovery are — treatment with activated charcoal, cation and anion-exchangers and crystallization.

Citric acid monohydrate formed below 36°C is the main commercial product. Above 40°C, citric acid crystallizes in an anhydrous form. The degree of purity of citric acid produced depends on the purpose for which it is required. For instance, pure forms of citric acid are needed for use in food preparations, while for industrial use it can be crude form.

2. Gluconic Acid:

Gluconic acid can be produced by several bacteria and fungi. Glucose, on a simple direct dehydrogenation, forms D-gluconolactone which is then converted to gluconic acid.

Applications of Gluconic Acid:

1. Gluconic acid is used in the manufacture of metals, stainless steel and leather, as it can remove the calcareous and rust deposits.

2. It is used as an additive to foods and beverages.

3. Gluconic acid has pharmaceutical applications — calcium and iron therapy.

4. Sodium gluconate is used as a sequestering agent in many detergents.

5. Gluconate is used for desizing polyester or polyamide fabrics.

6. It is utilized in the manufacture of highly resistant (to frost and cracking) concrete.

Microbial Production of Gluconic Acid:

Gluconic acid can be produced by a wide variety of prokaryotic and eukaryotic microorganisms.

Bacterial species of the genera— Gluconobacter, Acetobacter, Pseudomonas, Vibrio.

Fungal species of the genera—Aspergillus, Penicillium, Gliocladium.

Principle of production:

The enzymatic reactions for the formation of gluconic acid in Gluconobacter suboxidans (bacteria) and Aspergillus niger (fungus) are depicted in Fig. 24.6.

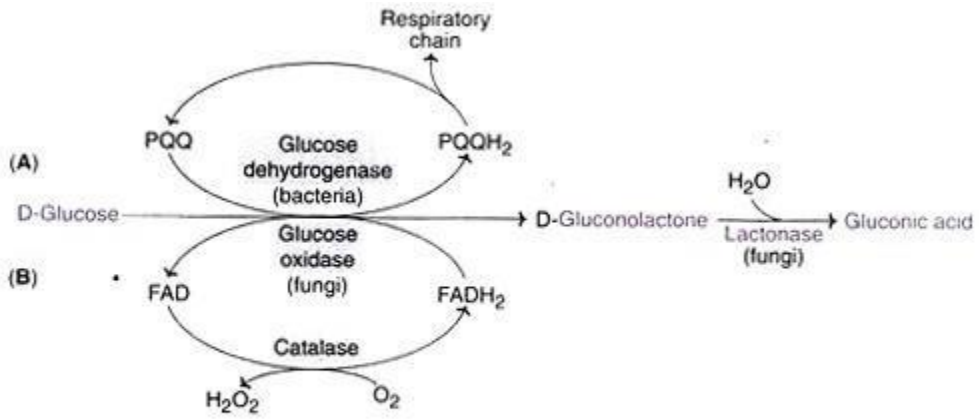


Fig. 24.6 : Biosynthesis of gluconic acid (A) in Gluconobacter suboxidans *(B) in* Aspergillus niger. *(PQQ-Pyrroloquinoline quinone)*

In bacteria, intracellular glucose is converted to extracellular gluconic acid. A membrane bound enzyme, glucose dehydrogenase utilizes pyrroloquinoline Quinone (PQQ) as coenzyme and converts glucose to 5-D-gluconolactone which undergoes hydrolysis (spontaneous or enzymatic) to form gluconic acid.

As regards fungal production, glucose is oxidized by the extracellular enzyme glucose oxidase to form 8-D-gluconolactone, which subsequently gets converted to gluconic acid by lactonase. Glucose oxidase is an inducible enzyme that can be induced by high concentrations of glucose, and at pH above 4. It is believed that H_2O_2 produced by glucose oxidase acts as an antagonist against other microorganisms (antimicrobial activity) in the surroundings.

Production Process for Gluconic Acid:

Submerged processes, by employing either A. niger or G. suboxidans, are used for producing gluconic acid. The culture medium contains glucose at a concentration of 12-15% (usually obtained from corn). The fermentation is carried out at pH 4.5-6.5 and at temperature 28-30°C for a period of about 24 hours.

Increasing the supply of O_2 enhances gluconic acid yield. Biotechnologists exploit the fermentation process of gluconic acid for the production of the enzyme glucose oxidase, besides producing calcium gluconate and sodium gluconate.

Chemical synthesis of gluconic acid:

By employing the immobilized enzyme glucose oxidase, gluconic acid can also be produced.

3. Lactic Acid:

Lactic acid occurs in two isomeric forms i.e. L (+) and D (-) isomers, and as a racemic mixture (DL-lactic acid). The isolation of lactic acid from milk was done in 1798. It was the first organic acid produced by microorganisms in 1880. Today, lactic acid is competitively produced both by microbiological and chemical methods.

Applications of Lactic Acid:

There are different grades of lactic acid mainly based on the percentage of lactic acid. The grades and their applications are given in Table 24.2.

TABLE 24.2 Commercial grades of lactic acid along with their applications

Grade (% lactic acid)	*Application(s)*
Technical grade (20–50%)	Ester manufacture, textile industry
Food grade (>80%)	Food additive (sour flour and dough)
Pharmaceutical grade (>90%)	Intestinal treatment (metal ion lactates)

Microorganisms for Production of Lactic Acid:

Lactic acid producing bacteria are broadly categorized into two types.

Hetero-fermentative bacteria—produce other byproducts, besides lactic acid, and therefore are not useful for industrial production of lactic acid. These bacteria are employed in food or feed preservation.

Homo-fermentative bacteria—specialised for exclusive production of lactic acid and therefore are suitable for industrial purpose.

Lactobacillus sp are used for lactic acid production. However, there are variations in the substrates utilised as indicated below.

L. delbrueckii, *L. leichamanni* } Glucose

L. bulgaricus, *L. helvetii* } Whey (lactose)

L. lactis — Maltose

L. amylophilus — Starch

L. pentosus — Sulfite waste liquor

Biosynthesis of lactic acid:

The synthesis of lactic acid occurs through glucose oxidation by glycolysis to produce pyruvate which on reduction gives lactic acid. The reducing equivalents ($NADH^+ + H^+$) produced during the oxidation of glyceraldelyde 3-phosphate are utilised by .the enzyme lactate dehydrogenase to form lactate (Fig. 24.7). Most of the lactic acid producing microorganisms normally produce only one isomer of lactic acid L(+) or D(-). However, some bacteria which usually occur as infection can form racemic mixture.

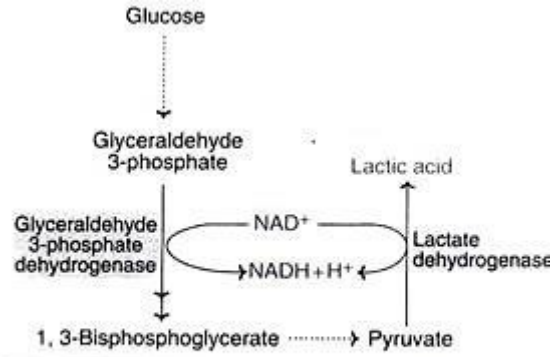


Fig. 24.7 : An outline of the pathway (glycolysis) for the biosynthesis of lactic acid.

Production Process for Lactic Acid:

The fermentation medium contains 12-15% of glucose, nitrogen and phosphate containing salts and micronutrients. The process is carried out at pH 5.5-6.5 and temperature 45-50°C for about 75 hours. Generally, the strains operating at higher temperature (45-60°C) are preferred, since it reduces the need for medium sterilization.

As the lactic acid is produced, it has to be removed since it is toxic to the organisms. This can achieved either by a continuous culture technique or by removal of lactic acid by electro dialysis. Theoretically, every molecule of glucose forms two molecules of lactic acid. About 90% of theoretical yield is possible in fermentation industry. L(+) Lactic acid is predominantly produced. The outline of the steps involved in the recovery lactic acid is depicted in Fig. 24.8.

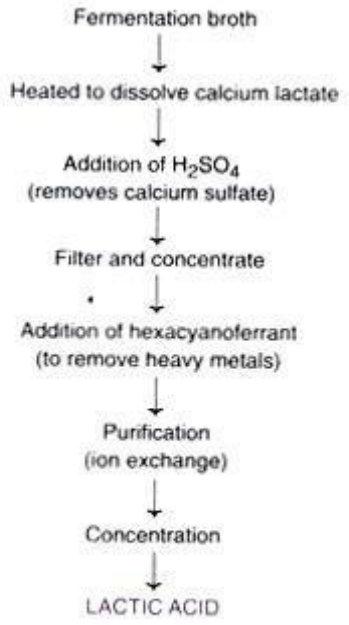


Fig. 24.8 : Flow chart for recovery of lactic acid from fermentation broth.

4. Acetic Acid:

The production of acetic acid, in the form of vinegar (used as a refreshing drink), from alcoholic liquids has been known for centuries.

Microorganisms Used for Production of Acetic Acid:

The commercial production of acetic acid is carried out by a special group of acetic acid bacteria, which are divided into two genera.

Gluconobacter that oxidizes ethanol exclusively to acetic acid.

Acetobacter that oxidizes ethanol first to acetic acid, and then to CO_2 and H_2O. These over-oxidizers are Gram-negative and acid tolerant e.g. A. aceti, A. peroxidans, A. pasteurianus.

Biosynthesis of acetic acid:

Acetic acid is a product of incomplete oxidation of ethanol. Ethanol is first oxidized by alcohol dehydrogenase to acetaldehyde which then gets hydrated to form acetaldehyde hydrate. The latter is then acted upon by acetaldehyde dehydrogenase to form acetic acid (Fig. 24.9).

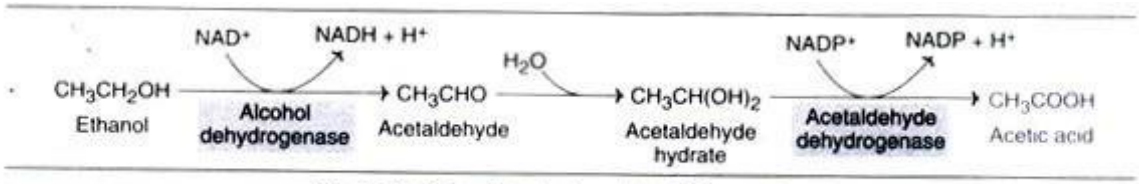


Fig. 24.9 : Biosynthesis of acetic acid from ethanol.

Production Process for Acetic Acid:

For every molecule of ethanol oxidised, one molecule of acetic acid is produced. Thus, high-yielding strains can produce 11-12% acetic acid from 12% alcohol. For optimal production, adequate supply of oxygen is very essential. Insufficient O_2, coupled with high concentration of alcohol and acetic acid result in the death of microorganisms. Surface fermentation or submerged fermentation processes can be carried out to produce acetic acid. Trickling generation process, a type of surface fermentation, is very commonly used.

Recovery:

The acetic acid produced is clarified by filtration and then subjected to decolourization by $K_4(FeCN)_6$.

Production of Vinegar:

Vinegar is an aqueous solution containing about 4% by volume acetic acid and small quantities of alcohol, salts, sugars and esters. It is widely used as a flavoring agent for processed liquid foods such as sauces and ketchups. The starting materials for vinegar production are wine, whey, malt (with low alcohol content). Vinegar production can be carried out either by surface process (trickling generator) or by submerged process.

Surface process:

The fermentation material is sprayed over the surface which trickles through the shavings that contain the acetic acid producing bacteria. The temperature is around 30°C on the upper part while it is around 35°C on the lower part. Vinegar is produced in about 3 days.

Submerged process:

The fermentation bioreactors are made up of stainless steel. Aeration is done by a suction pump from the top. The production rate in the submerged process is about 10 times higher than the surface process.

5. L-Ascorbic Acid:

L-Ascorbic acid is the commonly used chemical name for the water soluble vitamin C. This vitamin forms a redox system and participates in several biological processes. It is intimately involved in the biosynthesis of collagen, the most abundant protein in the human body. Vitamin C also protects the body against carcinogenic nitrosamines and free radicals. The deficiency of ascorbic acid causes scurvy.

Applications of Ascorbic Acid:

Because of the wide range of physiological and beneficial functions of ascorbic acid, its commercial production assumes significance. Vitamin C is mainly used in food and pharmaceutical industries.

Industrial Production of Ascorbic Acid:

Ascorbic acid is commercially produced by a combination of several chemical steps, and one reaction of biotransformation brought out by microorganisms. This process is referred to as Reichstein-Grussner synthesis (Fig. 24.10B). D-Glucose is first converted to D-sorbitol. Oxidation of D-sorbitol to L-sorbose is carried out by Acetobacter xylinum or A. suboxydans (The enzyme being sorbitol dehydrogenase).

A submerged bioreactor fermentation process is ideal for this reaction. It takes about 24 hours at temperature 30-35°C. Sorbose by a couple of chemical reactions can be finally converted to L-ascorbic acid. Normally, about 100 g of ascorbic acid is produced from 200 g of glucose in Reichstein-Grussner synthesis.

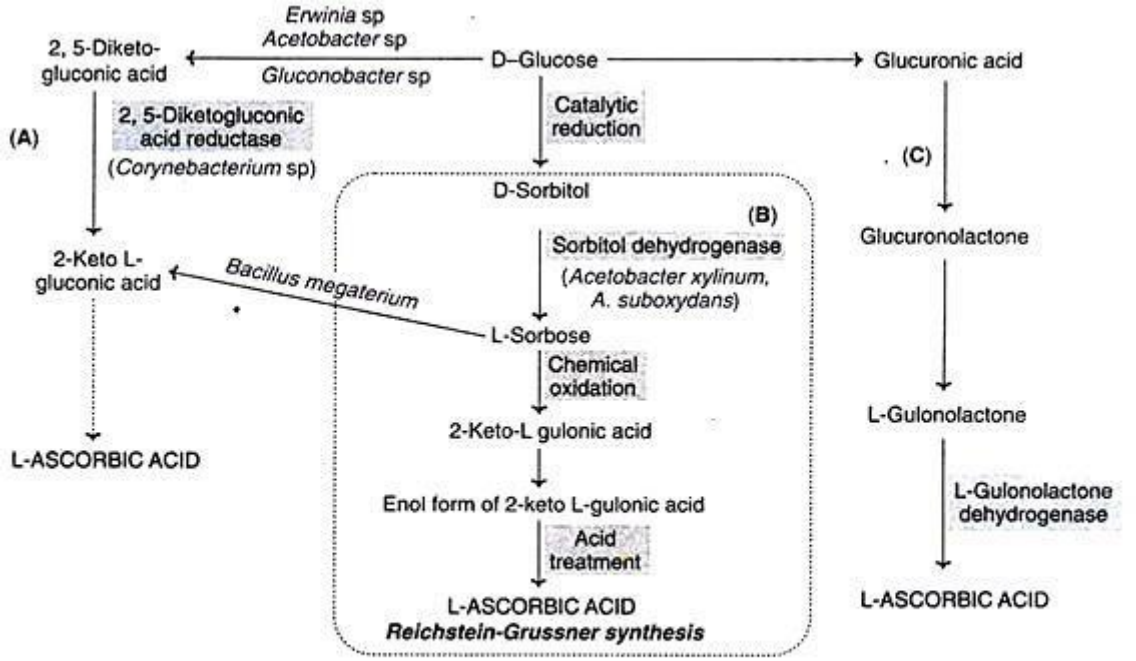


Fig. 24.10 : Pathways for the commercial production of ascorbic acid. (A) Two-step fermentation process (B) Reichstein-Grussner synthesis (C) Production via L-gulonolactone.

Two-step fermentation process:

In this, D-glucose is converted to 2, 5-diketogluconic acid by Erwinia, Acetobacter or Gluconobacter sp. In the second step, Corynebacterium sp converts 2, 5-diketogluconic acid to 2-keto-L-gluconic acid, (Fig. 24.10A). It is also possible to involve Bacillus megaterium for converting L-sorbose to 2-keto-L- gluconic acids. The latter, by chemical reactions, can be converted to ascorbic acid.

Production via L-gulonolactone:

Ascorbic acid can also be synthesized via- gulonolactone which can be directly converted to L-ascorbic acid by the enzyme L-gulonolactone dehydrogenase (Fig. 24.10C).

Direct Production of Ascorbic Acid by Fermentation:

Several workers are trying to produce ascorbic acid directly from glucose. Microalgae of Chlorella have shown some promising results, although the yield is very low.

Genetic Engineering for Ascorbic Acid Production:

Biotechnologists have been successful in cloning and expressing the gene for 2, 5-diketogluconic acid reductase of Corynebacterium sp into Erwinia herbicola. By doing this, the two step fermentation process (Fig. 24.10A) has been reduced to one. The genetically engineered Erwinia cells were able to convert D-glucose directly to 2-keto-L-gluconic acid.

This is certainly advantageous since the metabolic capabilities of two different micro-organisms could be combined into one organism. However, the yield of ascorbic acid by the hybrid strain was very low. Scientists are now trying to alter certain amino acids in 2-5 diketogluconic acid reductase and increase the catalytic activity of this enzyme.

6. Itaconic Acid:

Itaconic acid is used in plastic industry, paper industry and in the manufacture of adhesives. Itaconic acid can be commercially produced by Aspergillus itoconicus and A. terreus. The biosynthesis of itaconic acid occurs by way of Krebs cycle. The metabolite cis-aconitic acid (formed from citric acid) undergoes decarboxylation catalysed by the enzyme cis-aconitic decarboxylase (Fig. 24.11).

Itaconic acid is oxidised to itatartaric acid by itaconic acid oxidase. This enzyme has to be inhibited for a maximum yield of itaconic acid. This can be achieved by adding calcium. Batch submerged fermentation process is commonly used for itaconic acid production. The yield is around 75% of the theoretical calculation when the medium contains 15% sucrose.

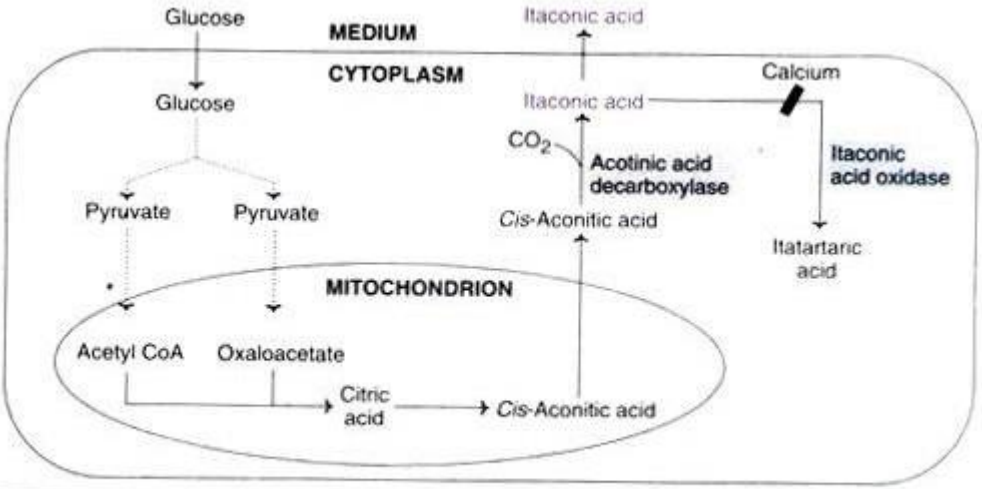


Fig. 24.11 : Biosynthesis of itaconic acid—an outline of metabolic pathway.

the microbial production of 7 types of amino acids. The seven types are: (1) Amino Acid (2) L-Glutamic Acid (3) L-Lysine (4) L-Threonine (5) L-Phenylalanine (6) L-Tryptophan and (7) L-Aspartic Acid.

Type # 1. Amino Acid:

Some general considerations on the production methods, and the development of strains of microorganisms for improved amino acid production are briefly described.

Methods for Production of Amino Acids:

The industrial production of amino acids is carried out by one or more of the following three processes:

1. Extraction:

Amino acids are the building blocks in protein structure. The proteins can be subjected to hydrolysis, and the requisite amino acids can be isolated e.g. cysteine, tyrosine, leucine.

2. Chemical synthesis:

Chemical synthesis results in a mixture of D- and L-amino acids. Most of the amino acids required for commercial applications are of L-category. However, for the synthesis of glycine (optically inactive) and some other amino acids which can be used in L- or D-form (D, L-alanine, D, L-methionine) for certain purposes, chemical methods are employed.

3. Microbiological production:

For the large- scale production of amino acids, microbiological methods are employed. There are three different approaches.

(a) Direct fermentation methods:

Amino acids can be produced by microorganisms by utilizing several carbon sources e.g. glucose, fructose, alkanes, ethanol, glycerol, propionate. Certain industrial byproducts like molasses and starch hydrolysate can also be used. Methanol, being a cheap carbon source, is tried for amino acid production, but with limited success.

(b) Conversion of metabolic intermediates into amino acids:

In this approach, the microorganisms are used to carry out selected reactions for amino acid production e.g. conversion of glycine to serine.

(c) Direct use of microbial enzymes or immobilized cells:

Sometimes resting cells, immobilized cells, crude cell extracts or enzyme-membrane reactors can be used for the production of amino acids. Some examples are given below. Amino acid dehydrogenases from certain bacteria (e.g. Bacillus megaterium) can be used for the amination of α-keto acids to produce L-amino acids e.g. alanine (from pyruvate), leucine (from α-ketoisocaproic acid) and phenylalanine (from phenyl pyruvate). Immobilized cells or

enzyme- membrane reactors can be used. Enzymes or immobilised cells are also employed for the production of several other amino acids e.g. tryptophan, tyrosine, lysine, valine.

Strain Development for Amino Acid Production:

The metabolic pathways, for the synthesis of amino acids by microorganisms, are tightly controlled and they operate in an economical way. Therefore, a natural overproduction of amino acids is a rare occurrence. Some strains that excrete certain amino acids have been isolated e.g. glutamic acid, alanine, valine.

In order to achieve an overproduction of any amino acid by a microorganism, methods have to be devised for the elimination of the metabolic regulatory/control processes. In fact, several amino acid-producing microorganisms have been developed by mutagenesis and screening programmes.

The following are the major ways of strain development. In fact, several methods are combined to successfully develop a new strain for producing amino acids.

Auxotrophic mutation:

These mutants are characterized by a lack of the formation of regulatory end product (i.e. repressor or regulatory effector). The intermediates of the metabolic pathways accumulate and get excreted.

Genetic recombination:

Mutants can be developed by genetic recombination for overproduction of amino acids. Protoplast fusion in certain bacteria is used for development of hybrids e.g. Corynebacterium glutamicum and Bacillus flavum.

Recombinant DNA technology:

The classical techniques of genetic engineering can be used for strain development. Strains with increasing activities of rate-limiting enzymes have been developed. In one of the techniques, E: coli and cloning vector pBR322 were used to increase the genes for the production of amino acids e.g. glutamic acid, lysine, phenylalanine, valine.

Functional genomics: a new approach:

Analysis of genomes from the wild and mutant strains of microorganisms will help in creating improved strains. Once the entire sequence of the chromosomes in the organisms (e.g. C. glutamicum, E. coli) is established, efforts can be made to carry out genetic manipulations for efficient overproduction of desired amino acids. Chip technology can be used to detect new mutations and consequently the fermentation processes.

Type # 2. L-Glutamic Acid:

L-Glutamic acid was the first amino acid to be produced by microorganisms. The original bacterium, Corynebacterium glutamicum, that was first used for large scale manufacture of glutamic acid continues to be successfully used even today. The other important organisms (although used to a lesser extent due to low yield) employed for glutamic acid production belong to genera Micro bacterium, Brevibacterium and Arthrobacter.

All these organisms have certain morphological and physiological characters comparable to C. glutamicum. Biochemically, glutamic acid- producing bacteria have a high activity of glutamate dehydrogenase and a low activity of α-ketoglutarate dehydrogenase. They also require the vitamin biotin.

Improved Production Strains:

Several improvements have been made, particularly in C. glutamicum, for improving the strains to produce and excrete more and more of glutamic acid. These include the strains that can tolerate high concentrations of biotin, and lysozyme-sensitive mutants with high yield.

Biosynthesis of L-glutamic Acid:

The pathway for the synthesis of glutamic acid with glucose as the carbon source is depicted in Fig. 26.1. Glucose is broken down to phosphoenol pyruvate and then to pyruvate. Pyruvate is converted to acetyl CoA. Phosphoenol pyruvate (by the enzyme phosphoenol pyruvate carboxylase) can be independently converted to oxaloacetate. Both these carboxylation reactions are quite critical, and require biotin as the cofactor.

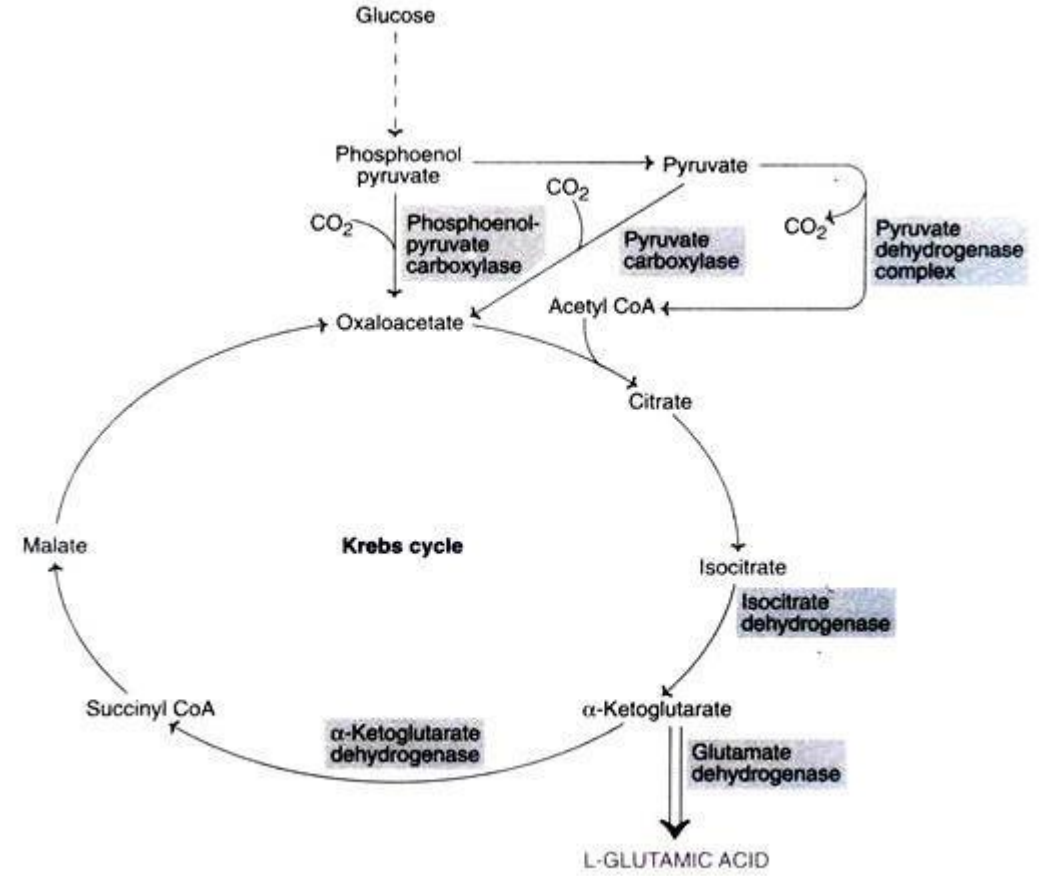


***Fig. 26.1 : Biosynthesis of L-glutamic acid in* Corynebacterium glutamicum.**

The next series of reactions that follow are the familiar citric acid (Krebs) cycle reactions wherein the key metabolite namely α-ketoglutarate is produced. In the routine citric acid cycle, α – ketoglutarate is acted upon by the enzyme α- ketoglutarate dehydrogenase to form succinyl CoA.

For the production of glutamic acid, α-ketoglutarate is converted to L-glutamic acid by the enzyme glutamate dehydrogenase (GDH). This enzyme is a multimer, each subunit with a molecular weight of 49,000. The reducing equivalents, in the form of NADPH + H^+, are required by GDH. They are generated in the preceding reaction of Krebs cycle (catalysed by the enzyme isocitrate dehydrogenase) while converting isocitrate to α-ketoglutarate. The supply and utilization of NADPH + H^+ occurs in a cyclic fashion through the participation of the two enzymes, namely isocitrate dehydrogenase and glutamate dehydrogenase (Fig. 26.2).

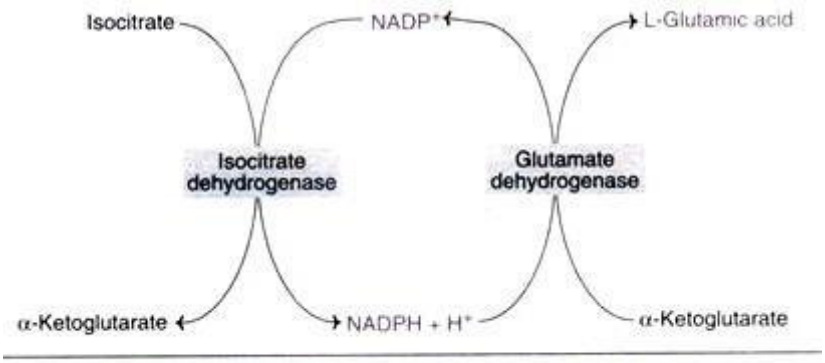


Fig. 26.2 : Biosynthesis of L. glutamic acid — role of NADP+.

Theoretically, one molecule of glutamic acid can be formed from one molecule of glucose. In practice, the conversion efficiency of glucose to glutamic acid was found to be around 70%.

Regulation of Glutamic Acid Biosynthesis:

The essential requirement for glutamic acid production is the high capability for the supply of the citric acid cycle metabolites. This is made possible by an efficient conversion of phosphoenol pyruvate as well as pyruvate to oxaloacetate .Thus, there are two enzymes (phosphoenol pyruvate carboxylase and pyruvate carboxylase) to efficiently produce oxaloacetate, while there is only one enzyme (pyruvate dehydrogenase) for the formation of acetyl CoA.

Certain microorganisms which have either phosphoenol pyruvate carboxylase (e.g., E. coli) or pyruvate carboxylase (e.g. B. subtilis) are not capable of producing glutamic acid to any significant extent. C. glutamicum has both the enzymes and therefore can replenish citric acid cycle intermediates (through oxaloacetate) while the synthesis of glutamic acid occurs.

Another key enzyme that can facilitate optimal production of glutamic acid is α-ketoglutarate dehydrogenase of citric acid cycle. Its activity has to be substantially low for good synthesis of glutamic acid, as is the case in C. glutamicum.

Further, exposing the cells to antibiotics (penicillin) and surfactants reduces the activity of α-ketoglutarate dehydrogenase while glutamate dehydrogenase activity remains unaltered. By this way, oxidation of α-ketoglutarate via citric acid cycle can be minimised, while the formation of glutamic acid is made maximum possible.

Release of Glutamic Acid:

Glutamic acid is synthesized intracellularly, and therefore its release or export is equally important. It now appears that there is a carrier-mediated energy-dependent active process involved for the export of glutamic acid.

There are several ways of increasing the membrane permeability for exporting glutamic acid:

i. Biotin limitation
ii. Addition of saturated fatty acids
iii. Addition of penicillin
iv. Use of oleic acid auxotroph's
v. Use of glycerol auxotroph's
vi. Addition of local anesthetics
vii. Addition of surfactants (Tween 40).

The effect of biotin deficiency in facilitating the release of intracellular glutamic acid has been worked out. Biotin is an essential cofactor (required by the enzyme acetyl CoA carboxylase) for the biosynthesis of fatty acids. Due to a limited supply or deficiency of biotin, fatty acid biosynthesis and consequently phospholipid synthesis is drastically reduced. As a result, membrane formation (protein- phospholipid complex) is defective which alters permeability for an increased export of intracellular glutamic acid.

It is found that there is an alteration in the membrane composition of phospholipids in oleic acid and glycerol auxotroph mutants. This facilitates release of intracellular glutamic acid. The knowledge on the membrane permeability of glutamic acid is successfully exploited for increased industrial production of glutamic acid.

Production of Glutamic Acid-Requirements and Influencing Factors:

The industrial production of glutamic acid is influenced by carbon sources, nitrogen sources, growth factors, pH and O_2 supply. The relevant aspects are briefly described.

Carbon sources:

Either refined (glucose, sucrose, fructose, maltose) or unrefined (sugar beet molasses, sugar cane molasses) carbon sources are used. In countries like Japan, acetate (inexpensive) is utilized. Other substrates like alkanes, ethanol and methanol are less frequently used.

Nitrogen sources:

The concentration of ammonia is very crucial for converting carbon source to glutamic acid. However, high concentration of ammonia inhibits the growth of the organisms. In the beginning of fermentation, ammonium salts and a low concentration of ammonia are added. During the course of fermentation, ammonia in aqueous solution is continuously fed. In this way, pH can be controlled, besides continuous supply of nitrogen source. Sometimes, urea is also used as a nitrogen source, since glutamic acid-producing bacteria possess urease that can split urea and release ammonia.

Growth factors:

Biotin is an important growth factor and its concentration in the medium is influenced by the carbon source. For instance, a supply 5 µg of biotin per liter medium is recommended if the carbon source is 10% glucose; while for acetate as the carbon source, the biotin requirement is much lower (0.1-1.0 µg/l). Addition of L-cysteine in the medium is recommended for certain strains.

Supply of O_2:

O_2 supply should be adequately and continuously maintained. It is observed that a high O_2 concentration inhibits growth of the organisms while a low O_2 supply leads to the production of lactic acid and succinic acid. In both instances, glutamic acid formation is low.

Process of Production and Recovery:

Some important information on the production of glutamic acid by Brevibacterium divaricatum is given below.

Carbon source – Glucose (12%)

Nitrogen source – Ammonium acetate (0.5%)

pH – 7.8

Temperature – 38°C

Period for fermentation – 30-35 hours

Yield of glutamic acid – 100 g/l medium.

A schematic representation of glutamic acid production plant is shown in Fig. 26.3. As the fermentation is complete, the cells are separated, the culture broth is passed through anion exchanger. The glutamic acid bound to the resins is eluted in NaOH, while the ammonia released can be reused. With NaOH, glutamic acid forms monosodium glutamate (MSG)

which can be purified by passing through anion exchanger. MSG can be subjected to evaporation and crystallization.

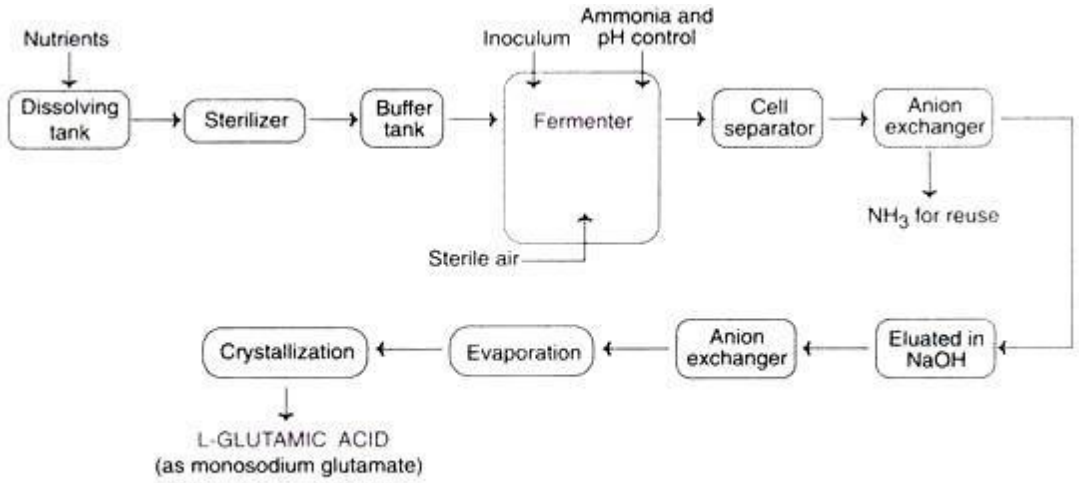


Fig. 26.3 : Diagrammatic representation of glutamic acid production plant.

Type # 3. L-Lysine:

Lysine is present at a low concentration in most of the plant proteins. Being an essential amino acid, supplementation of plant foods with lysine increases their nutritional quality. L-Lysine is predominantly produced by Corynebacterium glutamicum and to some extent by Brevibacterium flavum or B. lactofermentum.

Biosynthesis of L-lysine:

The pathway for the synthesis of L-lysine is complex, and an outline of it is depicted in Fig. 26.4. This metabolic pathway is also involved in the formation of 3 other amino acids, namely methionine, threonine and isoleucine.

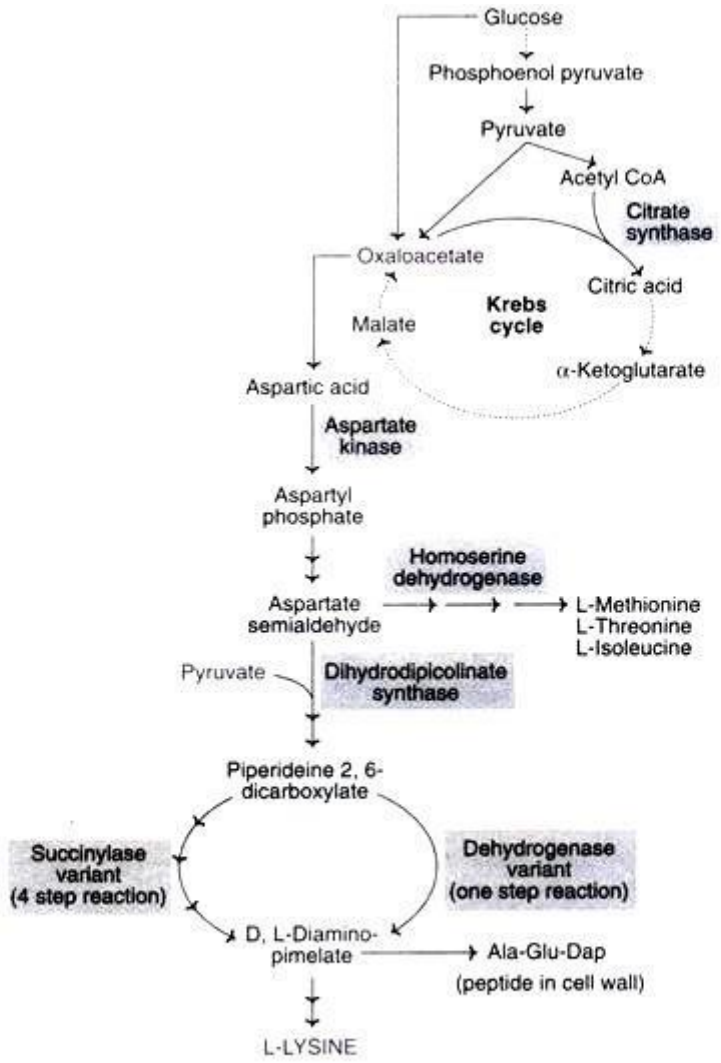


Fig. 26.4 : Biosynthesis of L-lysine in C. glutamicum
(Ala-Glu-Dap — Alanyl-glutamyl-diaminopimelate, a tripeptide)

As the glucose gets oxidised by glycolysis, phosphoenol pyruvate and pyruvate are formed. Both these metabolites can be converted to oxaloacetate, a key component of citric acid cycle. On transamination, oxaloacetate forms aspartate. The enzyme aspartate kinase converts aspartate to aspartyl phosphate which later forms aspartate semi-aldehyde.

Aspartate semi-aldehyde has two fates—the biosynthesis of lysine and formation of 3 other amino acids (methionine, threonine and isoleucine). When homoserine dehydrogenase acts on aspartate semi-aldehyde, it is diverted for the synthesis of 3 amino acids. The enzyme dihydrodipicolinate synthase converts aspartate semi-aldehyde (and pyruvate) to piperideine 2, 6-dicarboxylate.

There are two distinct enzymes succinylase variant (catalyses 4-step reaction) and dehydrogenase variant (catalyses a single step reaction) that can convert piperideine 2, 6-dicarboxylate to D, L-diaminopimelate which later forms L-lysine.

Regulation of L-lysine Biosynthesis:

The following are the regulatory processes in the production of lysine (Fig. 26.4).

Aspartate kinase:

This enzyme is controlled by feedback inhibition of the end products. Three isoenzymes of aspartate kinase have been identified-one repressed by L-methionine, the second one repressed by L-threonine and L-isoleucine, and the third one being inhibited and repressed by L-lysine. The amino acid sequence and structure of aspartate kinase have been elucidated. And by genetic manipulations, it has been possible to create mutants (of aspartate kinase) that are insensitive to feedback regulation by L-lysine.

Dihydrodipicolinate synthase:

This enzyme competes with homoserine dehydrogenase to act on aspartate semi-aldehyde. Overexpression of dihydrodipicolinate synthase has been shown to increase the production of L-lysine.

Succinylase and dehydrogenase variants:

The conversion of piperideine 2, 6-dicarboxylate to D, L-diaminopimelate is carried out by these two enzymes. At the start of the fermentation, dehydrogenase variant predominantly acts, and later succinylase variant comes into picture for the biosynthesis of L-lysine.

Role of D, L-diaminopimelate:

This amino acid, an immediate precursor for the synthesis of L-lysine, is also required for the synthesis of a tripeptide (L-Ala-γ-D-Glu-D, L-Dap) which is part of the peptidoglycan of cell wall. The activities of both the enzymes (succinylase and dehydrogenase) that form diaminopimelate (Dap) are important for the production of L-lysine and for the proper formation of cell wall structure.

Improved Production Strains:

Based on the biosynthetic pathway and the regulatory steps certain improvements have been made in the strains of C. glutamicum and B. flavum for overproduction of lysine.

i. Mutant organisms resistant to lysine antimetabolites (e.g. b-amino ethyl-L-lysine).

ii. A mutant strain with an altered enzyme aspartokinase, so that it is not regulated by end product inhibition.

iii. A strain with a decreased homoserine dehydrogenase activity (so that diversion for the synthesis of methionine, threonine and isoleucine is minimised).

iv. A strain with reduced citrate synthase activity (to lower the occurrence of citric acid cycle).

Release of L-Lysine:

The export or release of L-lysine from the cells into the surrounding medium occurs through a lysine-export (LysE) carrier protein. It is a trans membrane protein (mol. wt-25,400) with six segments that participate in lysine transport. The exporter system is very efficient active process to export large quantities of intracellular lysine.

Production Process of L-lysine:

The most commonly used carbon sources for lysine manufacture is molasses (cane or sugar beet), starch hydro lysates or sucrose. The other sources like acetate, ethanol or alkanes are used to a lesser extent. The nitrogen sources are ammonium salts, gaseous ammonia. Protein hydro lysates are added to supply certain amino acids (L-methionine, L-homoserine, L-threonine). The protein hydro lysates also supply growth factors such as biotin.

A time-course graphic representation for the formation of lysine is depicted in Fig. 26.5. As is evident, a continuous supply of glucose (or other sugar) is required for sustained production of lysine. Under optimal fermentation conditions, the yield of lysine (in the form of L-lysine HCI) is 40-50 g per 100 g carbon source.

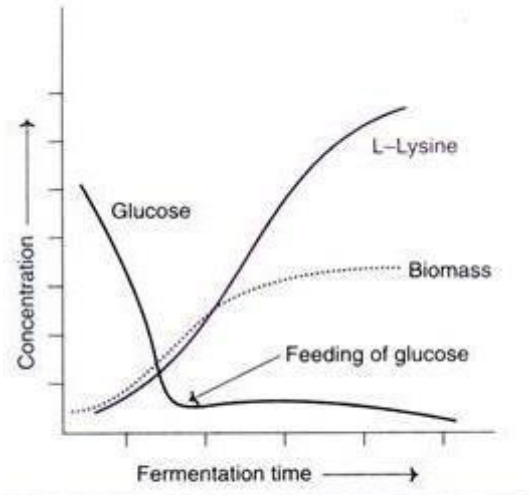


Fig. 26.5 : Production of L-lysine in relation to substrate (glucose) and biomass concentration.

There are different recovery processes for lysine depending on its application.

i. An alkaline solution containing about 50% L-lysine can be obtained after biomass separation, evaporation and filtration.

ii. A crystalline preparation with 98-99% L-lysine (as L-lysine HCI) can be obtained by subjecting the culture broth to ion-exchange chromatography, evaporation and crystallization.

Both the above grades of lysine are suitable for supplementation of feeds.

Type # 4. L-Threonine:

L-Threonine is manufactured industrially by employing either E. coli or C. glutamicum. With the mutant strains of E. coli, the product yield is better.

Biosynthesis of L-threonine:

The metabolic pathway for the synthesis of L-threonine is depicted in Fig. 26.6. Some of the reactions of this pathway are common for the biosynthesis of L-lysine and methionine, besides isoleucine .Starting with aspartic acid, in a sequence of five steps, threonine is produced.

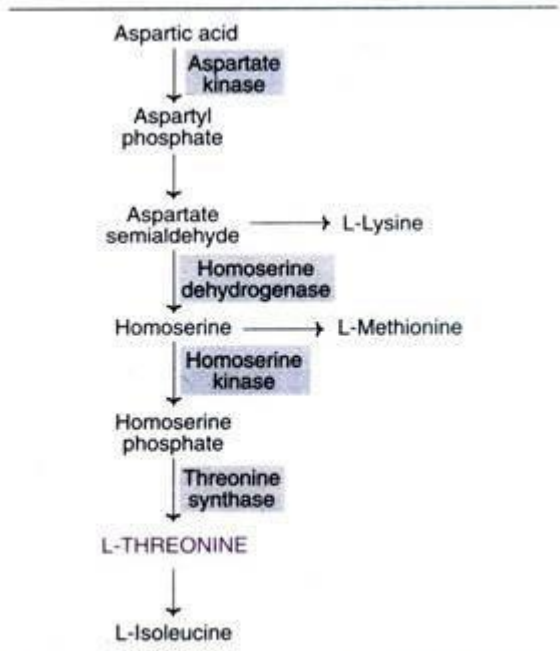


Fig. 26.6 : Biosynthesis of L-threonine in E. coli.

Regulation:

The regulatory reactions in E. coli for L-threonine biosynthesis have been elucidated. Three isoenzymes of aspartate kinase, separately inhibited by the end products have been identified-one by L-threonine, one by L-methionine and one by L-lysine.

Further, two isoenzymes of homoserine dehydrogenase-one inhibited by L-threonine and other by L-methionine are also known. A gene thrABC that encodes three polypeptides (one polypeptide possesses the activity of kinase and homoserine dehydrogenase, the second homoserine kinase and the third threonine synthase) in E. coli has been identified.

Improved production strains:

The efficiency of the producer strains can be increased by creating E. coli mutants with high-level expression of the gene thrABC. Further, mutants with minimal production of L-isoleucine also result is high yield of L-threonine.

Production Process of L-threonine:

The culture medium containing glucose or sucrose, yeast extract and ammonium salts is adequate for L-threonine production. The sugar feeding has to be continued for good yield (about 60% of the carbon source). The downstream processing for the isolation of L-threonine consists of coagulation of the cell mass (by heat), filtration, and concentration by evaporation, and crystallization.

Type # 5. L-Phenylalanine:

Both E. coli and C. glutamicum can be used for the production of L-phenylalanine. The biosynthetic pathway is quite complex and an outline is shown in Fig. 26.7. An interesting feature is that the same pathway is responsible for the synthesis of all the three aromatic amino acids-tyrosine and tryptophan, besides phenylalanine.

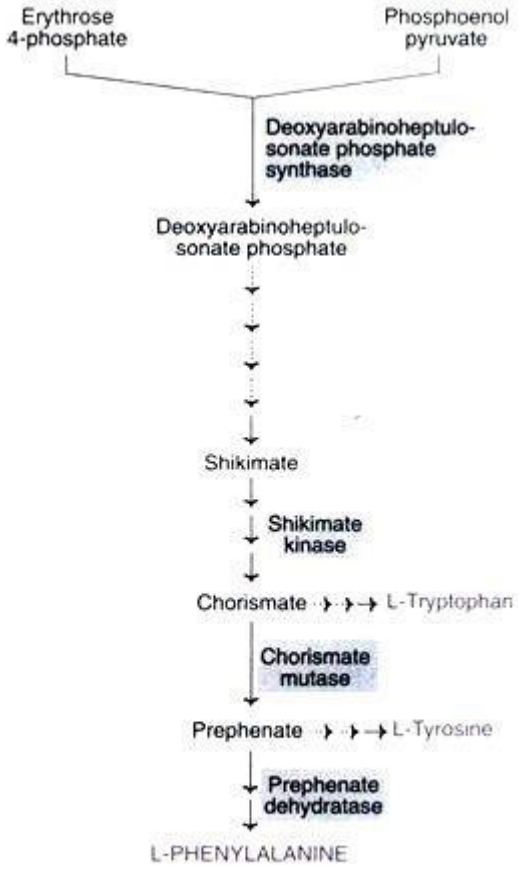


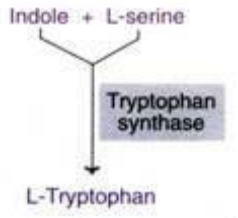
Fig. 26.7 : An outline of the pathway for the synthesis of L-phenylalanine, L-tyrosine and L-tryptophan.

The synthetic pathway commences with the condensation of erythrose 4-phosphate with phosphoenol pyruvate to form deoxyarabinoheptulosonate phosphate (DAHP). DAHP in the next series of reactions is converted to chorismate which can form L-tryptophan. Chorismate mutase converts chorismate to prephenate which forms L-phenylalanine through the participation of prephenate dehydrogenase. Prephenate also serves as a precursor for the synthesis of tyrosine.

The genes responsible for the formation of the regulatory enzymes of L-phenylalanine have been identified. By employing genetic manipulations, strains for improved production of L-phenylalanine have been developed.

Type # 6. L-Tryptophan:

There are different ways of synthesizing L-tryptophan-chemical, enzymatic and fermentation methods. At present, large scale manufacture of tryptophan is carried out by using the enzyme tryptophan synthase of E. coli. Tryptophan synthase combines indole with L-serine to form tryptophan.



Indole is available from petrochemical industries while L-serine can be recovered from molasses during sugar refinement. Mutant strains of E. coli with high activity of tryptophan synthase have been developed for large scale manufacture of tryptophan.

Direct fermentation process:

Tryptophan can also be produced by fermentation employing C. glutamicum, or E. coli. For the biosynthetic pathway, refer Fig. 26.7. Mutant strains of both these organisms have been developed for increased yield of tryptophan.

Mutant Strains for Overproduction L-tryptophan:

The production of tryptophan by C. glutamicum was increased by introducing a second gene encoding anthranilate synthase, a key enzyme in its biosynthesis (Fig. 26.8). Further, genes encoding other important enzymes (deoxyarabinoheptulosonate phosphate synthase, anthranilate phosphoribosyltransferase) were also be modified. The result is that the pathway becomes insensitive to feedback inhibition by end products, leading to an overproduction of L-tryptophan.

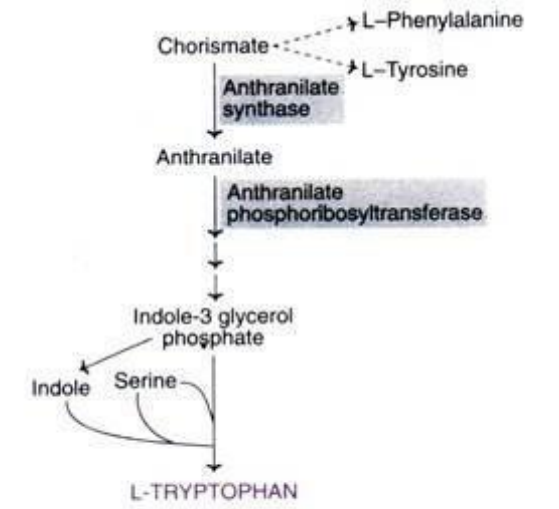
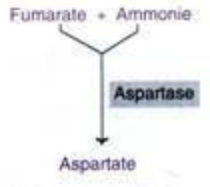


Fig. 26.8 : Biosynthesis of L-tryptophan (For the syntheis of chorismate Refer Fig. 26.7).

Type # 7. L-Aspartic Acid:

There is a growing demand for aspartate, as it is a component of aspartame (an artificial sweetener), besides its use as a food additive, and in pharmaceutical preparations. The preferred method for aspartate production is enzymatic in nature. The enzyme aspartase converts fumarate and ammonia to aspartate. Although this reaction is reversible, aspartate formation is favoured.



The aspartase of E. coli is used. It is a tetramer with a molecular weight 196,000. This enzyme is quite unstable. Immobilization of aspartase in polyacrylamide or carrageenan that enhances the stability of the enzyme is commonly used. Immobilized E. coli cells with good activity of aspartase are also used for aspartate production.

Microbial production of one of the organic feed stocks from plant substances such as molasses is presently used for ethanol production. This alcohol was produced by fermentation in the early days but for many years by chemical means through the catalytic hydration of ethylene.

In modem era, attention has been paid to the production of ethanol for chemical and fuel purposes by microbial fermentation. Ethanol is now-a-days produced by using sugar beet, potatoes, com, cassava, and sugar cane (Fig. 20.6).

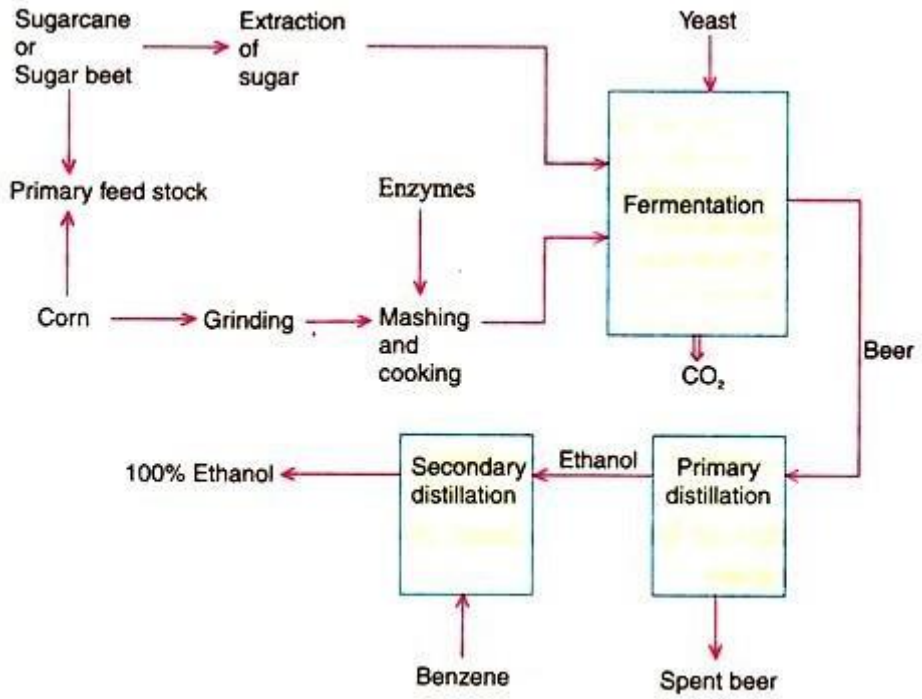


Fig. 20.6: Ethanol production from molasses.

Both yeasts (Saccharomyces cerevisiae, S. uvarum S. carlsbergensis, Candida brassicae, C. utilis, Kluyveromyces fragilis, K. lactis) and bacteria (Zymomonas mobilis) have been employed for ethanol production in industries.

The commercial production is carried out with Saccharomyces cerevisiae. On the other hand, uvarum has also largely been used. The Candida utilis is used for the fermentation of waste sulphite liquor since it also ferments pentoses.

Recently, experimentation with Schizosaccharomyces has shown promising results. When whey from milk is used, strain of K. fragilis is recommended for the production of ethanol. It

is also found that Fusarium, Bacillus and Pachysolen tannophilus (yeast) can transform pentose sugars to ethanol.

Theoretically, it is interesting to note that fermentation process retains most of the energy of the sugar in the form of ethanol. The heat of combustion of solid sucrose is 5.647 MJ mol-1, the heat of combustion of glucose is 2.816 MJ mol^{-1} but the heat release is 1.371 MJ mol-1.

The equations are given below:

1st equation: Because $C_{12}H_{24}O_{11}$ (sucrose) $\longrightarrow$ $4C_2H_5OH + 4CO_2$ (ethanol)

Hence, 5.647 $\longrightarrow$ $4 \times 1.371 = 5.184$

i.e. 97% conversion

2nd equation: In this case,

$C_6H_{12}O_6$ (glucose) $\longrightarrow$ $2C_2H_5OH + 2CO_2$ (ethanol)

Hence, 2.816 $\longrightarrow$ $2 \times 1.371 = 2.742$

i.e. 97% conversion

Thus the above reactions show that 97% sugar transforms into ethanol. But in practice, the fermentation yield of ethanol from sugar is about 46% or one hundred grams of pure glucose will yield 48.4 grams of ethanol, 46.6 g of CO_2, 3.3 grams of glycerol and 1.2 g of yeast. The biosynthesis of ethanol is given in Fig. 20.6.

It is noteworthy that the ethanol at high concentration inhibits the yeast. Hence, the concentration of ethanol reduces the yeast growth rate which affect the biosynthesis of ethanol.

It can produce about 10-12 % ethanol but the demerit of yeast is that it has limitation of converting whole biomass derived by their ability to convert xylulose into ethanol. The Zymomonas has a merit over yeast that it has osmotic tolerance to higher sugar concentration. It is relatively having high tolerance to ethanol and have more specific growth rate.

1. Preparation of Medium:

Three types of substrates are used for ethanol production:

(a) Starch containing substrate,

(b) Juice from sugarcane or molasses or sugar beet,

(c) Waste products from wood or processed wood. Production of ethanol from whey is not viable.

If yeast strains are to be used, the starch must be hydrolysed as yeast does not contain amylases. After hydrolysis, it is supplemented with celluloses of microbial origin so as to obtain reducing sugars. About 1 ton of starch required 1 litre of amylases and 3.5 litre of glucoamylases. Following steps are involved in conversion of starch into ethanol (Fig. 20.7).

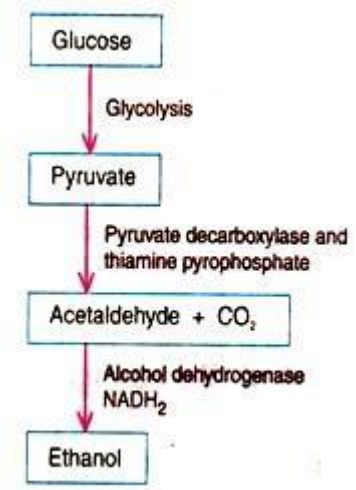


Fig. 20.7 : Biosynthesis of ethanol.

On the other hand, if molasses are used for ethanol production, the bagasse can also give ethanol after fermentation. Several other non-conventional sources of energy such as aquatic plant biomass, wood after hydrolysis with celluloses gives ethanol. Sulphite waste-liquor, a waste left after production of paper, also contains hexose as well as pentose sugar. The former can be microbially easily converted.

2. Fermentation:

Ethanol is produced by continuous fermentation. Hence, large fermenters are used for continuous manufacturing of ethanol. The process varies from one country to another. India, Brazil, Germany, Denmark have their own technology for ethanol production.

The fermentation conditions are almost similar (pH 5, temperature 35°C) but the cultures and culture conditions are different. The fermentation is normally carried out for several days but within 12h starts production. After the fermentation is over, the cells are separated to get biomass of yeast cells which are used as single cell protein (SCP) for animal's feed.

The culture medium or supernatant is processed for recovery of ethanol (Fig. 20.6). Ethanol is also produced by batch fermentation as no significant difference is found both in batch and continuous fermentation.

Although as stated earlier within 12h Saccharomyces cerevisiae starts producing ethanol at the rate of 10% (v/v) with 10-20g cells dry weight/lit. The reduction in fermentation time is accomplished use of ceil recycling continuously in fermentation.

3. Recovery:

Ethanol can be recovered upto 95 percent by successive distillations. To obtain 100 percent, it requires to form an azeotropic mixture containing 5 percent water. Thus 5 percent water is removed from azeotropic mixture of ethanol, water and benzene after distillation. In this procedure, benzene water ethanol and then ethanol-benzene azeotropic mixture are removed so that absolute alcohol is obtained.

Neuberg's Fermentation:

Yeasts utilize pyruvate during fermentation resulting in the formation of an intermediary product acetaldehyde.

This is trapped by hydrogen sulfite to yield the acetaldehyde in precipitated form and fluid product formation is glycerol as shown below:

$CH_3CHO + NaHSO_3 \rightarrow CH_2\text{-}CHOH\text{-}SO_3Na$

Now in place of acetaldehyde, dihydroxyacetone phosphate acts as a hydrogen acceptor which is reduced to glycerol-3-phosphate.

After removal of phosphate i.e. dephosphorylation, it gives glycerol as given below:

$C_6H_{12}O_6 + H_2SO_3 \rightarrow CH_2\text{-}CHOH\text{-}SO_3Na + Glycerol + CO_2$

Neuberg's fermentation process is categorized as reward and third fermentation.

The first fermentation equation is given below:

$2Glucose + H_2O \rightarrow C_2H_5OH + acetate + glycerol + 2CO_2$

PRODUCTION OF ANTIBIOTICS

Production of antibiotics is a naturally occurring event, that thanks to advances in science can now be replicated and improved upon in laboratory settings. Due to the discovery of penicillin by Alexander Fleming, and the efforts of Florey and Chain in 1938, large-scale, pharmaceutical **production of antibiotics** has been made possible. As with the initial discovery of penicillin, most antibiotics have been discovered as a result of happenstance. Antibiotic production can be grouped into three methods: natural fermentation, semi-synthetic, and synthetic. As more and more bacteria continue to develop resistance to currently produced antibiotics, research and development of new antibiotics continues to be important. In addition to research and development into the production of new antibiotics, repackaging delivery systems is important to improving efficacy of the antibiotics that are currently produced. Improvements to this field have seen the ability to add antibiotics directly into implanted devices, aerosolization of antibiotics for direct delivery, and combination of antibiotics with non antibiotics to improve outcomes. The increase of antibiotic resistant strains of pathogenic bacteria has led to an increased urgency for the funding of research and development of antibiotics and a desire for production of new and better acting antibiotics.

Despite the wide variety of known antibiotics, less than 1% of antimicrobial agents have medical or commercial value. For example, whereas penicillin has a high therapeutic index as it does not generally affect human cells, this is not so for many antibiotics. Other antibiotics simply lack advantage over those already in use, or have no other practical applications.

Useful antibiotics are often discovered using a screening process. To conduct such a screen, isolates of many different microorganisms are cultured and then tested for production of diffusible products that inhibit the growth of test organisms. Most antibiotics identified in such a screen are already known and must therefore be disregarded. The remainder must be tested for their selective toxicities and therapeutic activities, and the best candidates can be examined and possibly modified.

A more modern version of this approach is a rational design program. This involves screening directed towards finding new natural products that inhibit a specific target, such as

an enzyme only found in the target pathogen, rather than tests to show general inhibition of a culture.

Research into antibiotic identification has shown the opportunity exists to move away from lawn spotting methodology, a methodology which increases the chances of cross contamination. This new methodology involves using *Lactobacillus* species and shows a clear zone of inhibition as well as allowing for a determination of minimum inhibitory concentration.[1]

Industrial production techniques

Fermentation

Industrial microbiology can be used to produce antibiotics via the process of fermentation, where the source microorganism is grown in large containers (100,000–150,000 liters or more) containing a liquid growth medium. Oxygen concentration, temperature, pH and nutrient levels must be optimal, and are closely monitored and adjusted if necessary. As antibiotics are secondary metabolites, the population size must be controlled very carefully to ensure that maximum yield is obtained before the cells die. Once the process is complete, the antibiotic must be extracted and purified to a crystalline product. This is easier to achieve if the antibiotic is soluble in organic solvent. Otherwise it must first be removed by ion exchange, adsorption or chemical precipitation.

Semi-synthetic

A common form of antibiotic production in modern times is semi-synthetic. Semi-synthetic production of antibiotics is a combination of natural fermentation and laboratory work to maximize the antibiotic. Maximization can occur through efficacy of the drug itself, amount of antibiotics produced, and potency of the antibiotic being produced. Depending on the drug being produced and the ultimate usage of said antibiotic determines what one is attempting to produce.

An example of semi-synthetic production involves the drug ampicillin. A beta lactam antibiotic just like penicillin, ampicillin was developed by adding an addition amino group (NH_2) to the R group of penicillin. This additional amino group gives ampicillin a broader spectrum of use than penicillin. Methicillin is another derivative of penicillin and was discovered in the late 1950s, the key difference between penicillin and methicillin being the addition of two methoxy groups to the phenyl group.[These methoxy groups allow methicillin

to be used against penicillinase producing bacteria that would otherwise be resistant to penicillin.

Synthetic

Not all antibiotics are produced by bacteria; some are made completely synthetically in the lab. These include the quinolone class, of which nalidixic acid is often credited as the first to be discovered.Like other antibiotics before it the discovery of nalidixic acid has been chalked up to an accident, discovered when George Lesher was attempting to synthesize chloroquine. However a recent investigation into the origin of quinolones have discovered that a description for quinolones happened in 1949 and that patents were filed concerning quinolones some 5 years before Lesher's discovery

Strains used for the production[edit]

In the earliest years of antibiotic discovery the antibiotics being discovered were naturally produced antibiotics and were either produced by fungi, such as the antibiotic penicillin, or by soil bacteria, which can produce antibiotics including streptomycin and tetracycline.

Microorganisms used in fermentation are rarely identical to the wild type. This is because species are often genetically modified to yield the maximum amounts of antibiotics. Mutation is often used, and is encouraged by introducing mutagens such as ultraviolet radiation, x-rays or certain chemicals. Selection and further reproduction of the higher yielding strains over many generations can raise yields by 20-fold or more. Another technique used to increase yields is gene amplification, where copies of genes coding for enzymes involved in the antibiotic production can be inserted back into a cell, via vectors such as plasmids. This process must be closely linked with retesting of antibiotic production.

Some antibiotics are produced naturally by fungi. These include the cephalosporin producing *Acremonium chrysogenum.*

Geldanamycin is produced by Streptomyces hygroscopicus.

Erythromycin is produced by what was called Streptomyces erythreus and is now known as *Saccharopolyspora erythraea.*

Streptomycin is produced by *Streptomyces griseus*.

Tetracycline is produced by *Streptomyces aureofaciens*

Vancomycin is produced by Streptomyces orientalis, now known as *Amycolatopsis orientalis.*

Advancements

Penicillin was the first of the antibiotics to be discovered. After the discovery there was the issue of taking the raw naturally produced penicillin and developing a method so that wide-scale production of a clinically significant antibiotic could occur. Over the course of many years a team led by Florey and Chain and based in Oxford was able to successfully purify, concentrate, and produce the antibiotic.

Advances in scientific technology have not always led to better conditions for the production of antibiotics. Since 1987 there have been no new classes of antibiotics discovered for industrial production and widespread usage. However new developments in genomic sequencing and technology have led to improvements and discovery in the field of antibiotic production. Genomic engineering of antibiotic gene clusters has already been shown to lead to an increase in production of different antibiotics.

Antibiotic production

Antibiotics do not render themselves fully functional and deliverable simply by being produced. Often modifications must be made to the antibiotics so that maximum efficiency is attained. Post-production modifications include making antibiotics aerosolized so as to bypass doing unnecessary damage to bacteria located in other parts of the body and instead going directly to the lungs. Nosocomial infections can lead to serious complications during and in the recovery following surgery or a hospital stay in general. By merging surgical implants with antibiotics, healthcare providers are able to strike at a specific high risk area of infection without having to use a body wide size dosage of antibiotics.

Meropenem is an antibiotic that is delivered into the body via injection. When produced meropenem is a crystalline antibiotic, so it must be mixed in with solution before injection can occur. During this process meropenem is mixed with sodium carbonate, then diluted in water after which it can be injected.

Aerosolization of antibiotics is necessary because infections of the lung are especially troublesome, which is why direct targeting of the infection is needed. Broad spectrum antibiotics can have detrimental side effects when their action is also taken against necessary non-pathogenic bacteria residing in the human microbiome. Aerosolization is effective in bypassing the microbiome that exists in the gastrointestinal tract by directing the antibiotic directly to the lungs. This process is undertaken after the production of the antibiotic itself.

an overview on microbial production of vitamins.

Vitamins are organic compounds that perform specific biological functions for normal maintenance and optimal growth of an organism. These vitamins cannot be synthesized by the higher organisms, including man, and therefore they have to be supplied in small amounts in the diet.

Microorganisms are capable of synthesizing the vitamins. In fact, the bacteria in the gut of humans can produce some of the vitamins, which if appropriately absorbed can partially meet the body's requirements. It is an accepted fact that after administration of strong antibiotics to humans (which kill bacteria in gut), additional consumption of vitamins is recommended.

Microorganisms can be successfully used for the commercial production of many of the vitamins e.g. thiamine, riboflavin, pyridoxine, folic acid, pantothenic acid, biotin, vitamin B_{12}, ascorbic acid, P-carotene (pro-vitamin A), ergosterol (pro-vitamin D). However, from economic point of view, it is feasible to produce vitamin B_{12}, riboflavin, ascorbic acid and p-carotene by microorganisms. For the production of ascorbic acid (vitamin C), the reader must.

Vitamin B_{12}:

The disease, pernicious anemia, characterized by low levels of hemoglobin, decreased number of erythrocytes and neurological manifestations, has been known for several decades. It was in 1926 some workers reported the liver extracts could cure pernicious anemia. The active principle was later identified as vitamin B_{12}, a water soluble B-complex vitamin.

Occurrence:

Vitamin B_{12} is present in animal tissue at a very low concentration (e.g. 1 ppm in the liver). It occurs mostly in the coenzyme forms- methylcobalamin and deoxyadenosylcobalamin. Isolation of vitamin B_{J2} from animal tissues is very expensive and tedious.

Chemistry:

Vitamin B_{12} (cyanocobalamin) is a water soluble vitamin with complex structure. The empirical formula of cyanocobalamin is $C_{63}H_{90}N_{14}O_{14}PCO$. The structure of vitamin B_{12} consists of a corrin ring with a central cobalt atom. The corrin ring is almost similar to the

tetrapyrrole ring structure found in other porphyrin compounds e.g. heme (with Fe) and chlorophyll (with Mg).

The corrin ring has four pyrrole units. Cobalt present at the centre of the corrin ring is bonded to the four pyrrole nitrogen's. Cobalt also binds to dimethylbenzimidazole and amino isopropanol. Thus, cobalt atom present in vitamin B_{12} is in a coordination state of six.

Biosynthesis:

Vitamin B_{12} is exclusively synthesized in nature by microorganisms. An outline of the pathway is depicted in Fig. 27.1. The biosynthesis of B_{12} is comparable with that of chlorophyll and hemoglobin. Many of the reactions in the synthesis of vitamin B_{12} are not yet fully understood.

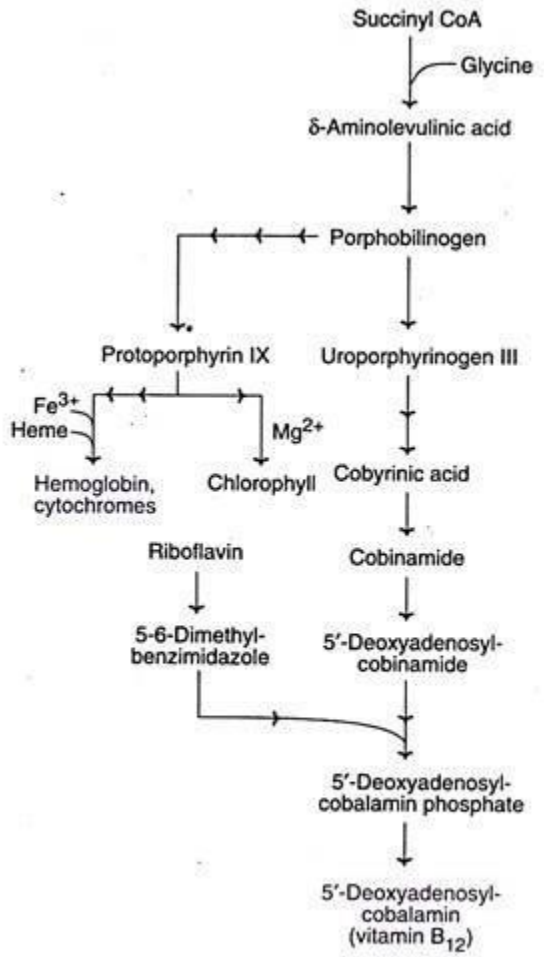


Fig. 27.1 : An outline of the biosynthesis of vitamin B_{12}

Commercial Production of Vitamin B_{12}:

Vitamin B_{12} is commercially produced by fermentation. It was first obtained as a byproduct of Streptomyces fermentation in the production of certain antibiotics (streptomycin, chloramphenicol, or neomycin). But the yield was very low. Later, high-yielding strains were developed. And at present, vitamin B_{12} is entirely produced by fermentation. It is estimated that the world's annual production of vitamin B_{12} is around 15,000 kg.

High concentrations of vitamin B_{12} are detected in sewage-sludge solids. This is produced by microorganisms. Recovery of vitamin B_{12} from sewage-sludge was carried out in some parts of United States. Unlike most other vitamins, the chemical synthesis of vitamin B_{12} is not practicable, since about 20 complicated reaction steps need to be carried out. Fermentation of vitamin B_{12} is the only choice.

Microorganisms and Yields of Vitamin B_{12}:

Several microorganisms can be employed for the production of vitamin B_{12}, with varying yields. Glucose is the most commonly used carbon source. Some examples of microbes and their corresponding yields are given in Table 27.1. The most commonly used microorganisms are — Propionibacterium freudenreichii, Pseudomonas denitrificans, Bacillus megaterium and Streptomyces olivaceus.

Table 27.1 Microorganisms with corresponding yields of vitamin B_{12}

Microorganism	*Yield (mg/l)*
Bacillus megaterium	0.51
Streptomyces olivaceus	3.31
Butyribacterium rettgeri	5.0
Micromonospora sp	11.5
Propionibacterium freudenreichii	19.0
Propionibacterium shermanii	35.0
Pseudomonas denitrificans	60.0
Hybrid strain	
Rhodopseudomonas protamicus	135.0

Genetically engineered strains for vitamin B_{12} production:

By employing modern techniques of genetic engineering, vitamin B_{12} production can be enhanced. A protoplast fusion technique between Protaminobacter rubber and Rhodopseudomonas spheroides resulted in a hybrid strain called Rhodopseudomonas

protamicus. This new strain can produce as high as 135 mg/l of vitamin B_{12} utilizing carbon source.

Production of Vitamin B_{12} Using Propionibacterium sp:

Propionibacterium freudenreichii and P. shermanii, and their mutant strains are commonly used for vitamin B_{12} production. The process is carried out by adding cobalt in two phases.

Anaerobic phase:

This is a preliminary phase that may take 2-4 days. In the anaerobic phase 5′-deoxyadenosylcobinamide is predominantly produced.

Aerobic phase:

In this phase, 5, 6-dimethyl- Benz imidazole is produced from riboflavin which gets incorporated to finally form coenzyme of vitamin B-p namely 5′-deoxyadenosylcobalamin. In recent years, some fermentation technologists have successfully clubbed both an anaerobic and aerobic phases to carry out the operation continuously in two reaction tanks.

The bulk production of vitamin B_{12} is mostly done by submerged bacterial fermentation with beet molasses medium supplemented with cobalt chloride. The specific details of the process are kept as a guarded secret by the companies.

Recovery of vitamin B_{12}:

The cobalamins produced by fermentation are mostly bound to the cells. They can be solubilized by heat treatment at 80-120°C for about 30 minutes at pH 6.5-8.5. The solids and mycelium are filtered or centrifuged and the fermentation broth collected. The cobalamins can be converted to more stable cyanocobalamins. This vitamin B_{12} is around 80% purity and can be directly used as a feed additive. However, for medical use (particularly for treatment of pernicious anemia), vitamin B_{12} should be further purified (95-98% purity).

Production of Vitamin B_{12} using Pseudomonas sp:

Pseudomonas denitrificans is also used for large scale production of vitamin B_{12} in a cost-effective manner. Starting with a low yield (0.6 mg/l) two decades ago, several improvements have been made in the strains of P. denitrificans for a tremendous improvement in the yield (60 mg/l). Addition of cobalt and 5, 6-dimethyl Benz imidazole to the medium is essential.

The yield of vitamin B_{12} increases when the medium is supplemented with betaine (usual source being sugar beet molasses).

Carbon Sources for Vitamin B_{12} Production:

Glucose is the most commonly used carbon source for large scale manufacture of vitamin B_{12}. Other carbon sources like alcohols (methanol, ethanol, isopropanol) and hydrocarbons (alkanes, decane, hexadecane) with varying yields can also be used. A yield of 42 mg/l of vitamin B_{12} was reported using methanol as the carbon source by the microorganism Methanosarcina barkeri, in fed- batch culture system.

Riboflavin:

Riboflavin (vitamin B_{12}) is a water soluble vitamin, essential for growth and reproduction in man and animals. Deficiency of riboflavin in rats causes growth retardation, dermatitis and eye lesions. In humans, vitamin B_2 deficiency results in cheilosis (fissures at the corner of mouth), glossitis (purplish tongue) and dermatitis. Riboflavin exerts its biochemical functions through the coenzymes namely flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN).

Occurrence:

Riboflavin occurs in milk and milk products, meat, eggs, liver and kidney. While in milk and eggs, it is present in free form, in other foods it is found in the form of flavoproteins (i.e. coenzymes of riboflavin bound to proteins).

Chemistry:

Riboflavin contains 6, 7-dimethyl isoalloxazine (a heterocyclic 3 ring structure) attached to D-ribitol by a nitrogen atom. The isoalloxazine ring participates in the oxidation-reduction reactions brought out by the coenzymes (FAD and FMN).

Biosynthesis:

The biosynthetic pathway of riboflavin, elucidated for the microorganisms Ashbya gossypii and Eremothecium ashbyii is depicted in Fig. 27.2. The overproduction of riboflavin in these organisms takes place mainly due to the constitutive nature of the riboflavin synthesizing enzymes. Iron which inhibits the production of vitamin B_{12} in Clostridia and yeasts, has no effect on A. gossypii and E. ashbyii.

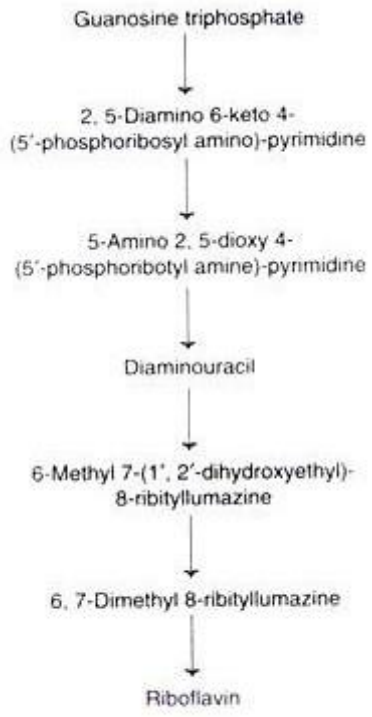


Fig. 27.2 : Biosynthesis of riboflavin.

Commercial Production of Riboflavin:

There are three processes employed for the large scale production of riboflavin. The worldwide requirement of riboflavin is estimated to be around 2,500 tones per year.

1. Biotransformation:

About 50% of the world's requirement of riboflavin is produced by biotransformation, followed by chemical synthesis. For this purpose, glucose is first converted to D-ribose by mutant strains of Bacillus pumilus. The D-ribose so produced is converted to riboflavin by chemical reactions.

2. Chemical synthesis:

Approximately 20% of the world's riboflavin is produced by direct chemical synthesis.

3. Fermentation:

At least one third of world's riboflavin requirements are met by direct fermentation processes.

Microorganisms and yields of riboflavin:

Several microorganisms (bacteria, yeasts and fungi) can be employed for the production of riboflavin. In the acetone-butanol fermentation, employing the organisms Clostridium aceto-butylicum and Clostridium butylicum, riboflavin is formed as a byproduct.

Commercial production of riboflavin is predominantly carried out by direct fermentation using the ascomycetes. The different organisms used and the corresponding yields of riboflavin are given in Table 27.2. The two plant pathogens namely Ashbya gossypii and Eremothecium ashbyii are most commonly employed due to high yield. Among these two organisms, A. gossypii is preferred as it is more stable with a high producing capacity of riboflavin.

TABLE 27.2 Microorganisms with corresponding yields of riboflavin

Microorganism	*Yield (mg/l)*
Clostridium acetobutylicum	0.097
Clostridium butylicum	0.120
Mycobacterium smegmatis	0.060
Mycocandida riboflavina	0.200
Candida flareri	0.575
Eremothecium ashbyii	2.500
Ashbya gossypii	7.500

Genetically engineered strains for riboflavin production:

High yielding strains of Ashbya gossypii have been developed by genetic manipulations. Such strains can yield as high as 15 g/1 riboflavin.

Production process of riboflavin:

Industrial production of riboflavin is mostly carried out with the organism, Ashbya gossypii by using simple sugars such as glucose and corn steep liquor. Glucose can be replaced by sucrose or maltose for the supply of carbon source.

In recent years, lipids such as corn oil, when added to the medium for energy purpose, have a profound influence on riboflavin production. Further, supplementation of the medium with yeast extract, peptones, glycine, inositol, purines (not pyrimidine's) also increase the yield of riboflavin.

It is essential to carefully sterilize the medium for good yield of riboflavin. The initial pH of the culture medium is adjusted to around 6-7.5. The fermentation is conducted at temperature

26-28°C with an aeration rate 0.3 vvm. The process is carried out for about 5-7 days by submerged aerated fermentation.

Riboflavin fermentation by Eremothecium ashbyii is comparable to that described above for Ashbya gossypii. Candida sp can also produce riboflavin, but this fermentation process is extremely sensitive to the presence of iron. Consequently, iron or steel equipment cannot be used. Such equipment have to be lined with plastic material.

Fermentation through phases:

Some studies have been carried out to understand the process of fermentation of riboflavin particularly by ascomycetes. It is now accepted that the fermentation occurs through three phases.

Phase I:

This phase is characterized by rapid growth of the organism utilizing glucose. As pyruvic acid accumulates, pH becomes acidic. The growth of the organism stops as glucose gets exhausted. In phase I, there is no production of riboflavin.

Phase II:

Sporulation occurs in this phase, and pyruvate concentration decreases. Simultaneously, there is an accumulation of ammonia (due to enhanced deaminase activity) which makes the medium alkaline. Phase II is characterized by a maximal production of riboflavin. But this is mostly in the form of FAD and a small portion of it as FMN.

Phase III:

In this last phase, cells get disrupted by a process of autolysis. This allows release of FAD, FMN and free riboflavin into the medium.

Recovery:

Riboflavin is found in fermentation broth and in a bound form to the cells. The latter can be released by heat treatment i.e. 120°C for about 1 hour. The cells can be discarded after filtration or centrifugation. The filtrate can be further purified and dried, as per the requirements.

Other carbon sources for riboflavin production:

Besides sugars, other carbon sources have also been used for riboflavin production. A pure grade of riboflavin can be prepared by using Saccharomyces sp, utilizing acetate as sole carbon source. Methanol-utilizing organism Hansenula polymorpha was found to produce

riboflavin. The other carbon sources used with limited success for riboflavin production are aliphatic hydrocarbons (organism Pichia guilliermoudii) and n-hexadecane (organisms — Pichia miso).

β– Carotene:

β- Carotene is the pro-vitamin A. When ingested, it gets converted to vitamin A in the intestine. Vitamin A is a fat soluble vitamin required for vision, proper growth and reproduction. The deficiency of vitamin A causes night blindness, changes in the skin and mucosal membranes.

Occurrence and chemistry:

β- Carotene is found in many animal and plant tissues. However, it originates exclusively from plants or microorganisms. Yellow and dark green vegetables and fruits are rich in β-carotene e.g. carrots, spinach, amaranthus, mango, papaya.

Carotenoids are isoprene derivatives. Chemically, they are tetraterpenoids with eight isoprene residues. There are around 400 naturally occurring carotenoids. The most important carotenoids are β-carotene, α-carotene, δ-carotene, lycopene and zeaxanthin.

Carotenoids are mainly used as colouring agents e.g., β-carotene, lycopene, xanthophyll's. Several foods (cheese, meat, egg products) can be made attractive by coloration. It may be noted that the demand for β-carotene as the pro-vitamin A is comparatively less.

Biosynthesis:

The pathway for the biosynthesis of β-carotene and some other important carotenoids, elucidated in plants and fungi, is shown in Fig. 27.3.

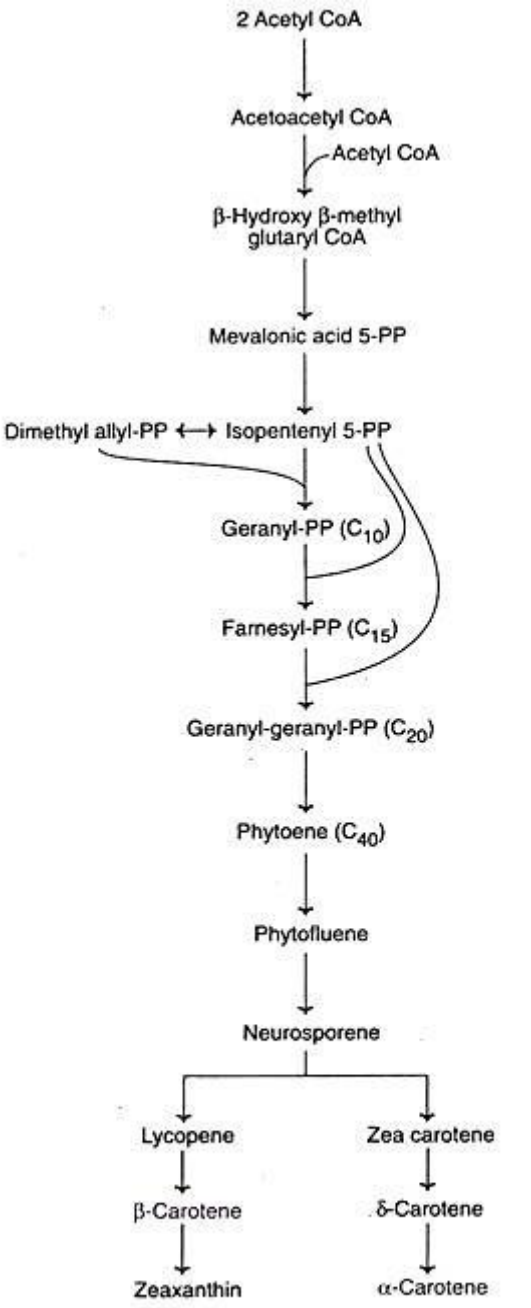


Fig. 27.3 : Biosynthesis of carotenes.

Commercial Production of β-Carotene:

β- Carotene can be produced by microbial fermentation. However, for economic reasons, direct chemical synthesis of vitamin A is preferred rather than using its pro-vitamin (β-carotene).

Microorganisms:

The organisms Blakeslea trispora, Phycomyces blakesleeanus and Choanephora cucurbitarum are most frequently used for the production of β– carotene. Among these, Blakeslea trispora is preferred due to high yield. In the Table 27.3, some important carotenoids, the organisms and the production yields are given.

TABLE 27.3 Microbial production of important carotenoids

Carotenoid	*Organism*	*Production yield (g/l)*
β-Carotene	*Blakeslea trispora* (mixed cultures of + and – sexual forms)	3.0
Lycopene	*Blakeshlea trispora* (mixed culture)	0.4
	Streptomyces chrestomyceticus	0.5
Zeaxanthin	*Flavobacterium* sp	0.4

Production process of β-carotene:

As already stated, the industrial production of β-carotene is mostly carried out by Blakeslea trispora. The fermentation medium contains corn starch, soybean meal, β-ionone, antioxidants etc. Addition of antioxidants improves the stability of β-carotene within the cells. The fermentation is carried out by submerged process.

The fermentation is usually started by mixing the cultures of both sexual forms, (+) and (-) strains of B. trispora. The yield of β-carotene is significantly higher with mixed cultures, compared to + or – strains (Fig. 27.4).

This is due to the fact that β- carotene production predominantly occurs during the process of zygospore formation. It may be stated here that the use of mixed strains does not improve the yield for other microorganisms (as observed in case of Blakeslea trispora).

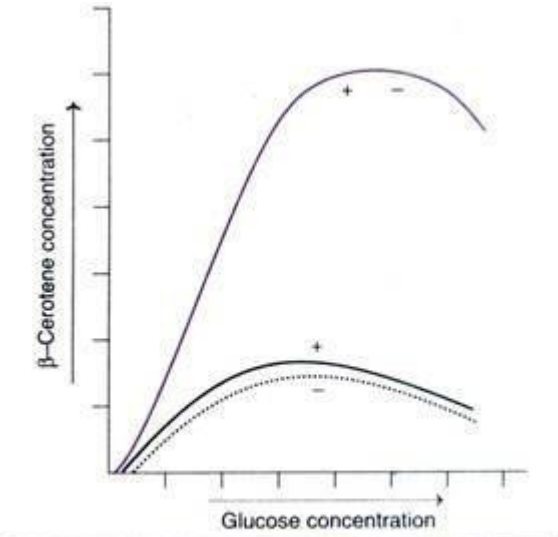


Fig. 27.4 : Yield of β–carotene by +, – and mixed (+ –) cultures of Blakesba trispora.

Factors affecting production:

Trisporic acid which can act as a microbial sexual hormone improves production yield of β-carotene. β-lonones enhance p-carotene synthesis by increasing the activity of enzymes, and not by their direct incorporation into β-carotene. When the fermentation medium is supplemented with purified kerosene, β-carotene production is almost doubled. Kerosene increases the solubility of hydrophobic substrates.

Recovery:

The mycelium rich in β-carotene can be directly used as a feed additive. For purification, mycelium is removed, subjected to dehydration (by methanol) and extracted in methylene chloride. This product is of 70-85% purity which can be further purified as per the requirements.

Gibberellins — Plant Growth Stimulants:

Gibberellins are plant hormones that stimulate plant growth. They promote growth by cell enlargement and cell division. The observable effects of gibberellins include stimulus to seed germination, flowering and lengthening of stems.

Microbial Production of Gibberellins:

So far only one microorganism, the fungus namely Gibberella fujikuroi has been found to produce gibberellins. This is actually a pathogenic fungus of rice seedlings. Gibberellin production can be carried out by using a glucose-salt medium at pH 7.5 and temperature

25°C for 2-3 days. The fermentation process is conducted in aerated submerged process. After the growth of the fungus is maximum, the production of gibberellins commences.

Biotransformation of Steroids:

All the steroids possess the basic structure namely cyclopentanoperhydrophenanthrene. Steroids as hormones (glucocorticoids, mineralocorticoids, androgens, estrogens) perform a wide range of functions. They are very useful therapeutically. For instance, cortisone, due to its anti-inflammatory action is used in the treatment of rheumatoid arthritis and skin diseases; derivatives of progesterone and estrogens are employed as contraceptives. Certain derivatives of cortisone (e.g. prednisolone) are more effective in their therapeutic action.

Commercial production of steroids is very important. Cortisone was chemically synthesized, and this process involved as many as 37 reactions. The cost of the so obtained product was around $200/g (in 1950). With the introduction of biotransformation reactions, the number of steps (microbial and chemical put together) was reduced to II, and cost of the product was reduced to just $1/g in 1980! The credit obviously goes to the developments in biotransformation.

Types of reactions in biotransformation of steroids:

The microbial transformation of steroids broadly involves oxidation (introduction of hydroxyl groups, splitting of side chains, production of epoxides etc.) reduction (conversion of aldehydes or ketones to alcohols, hydration of double bonds), hydrolysis and ester formation.

Production process of steroids:

The production of steroids, entirely by biotransformation reactions is not practicable. Therefore, microbial transformation along with chemical reactions is carried out. The major steps involved in the biotransformation of steroids are depicted in Fig. 22.1. Stigma sterol extracted from soybeans or diosgenin isolated from the roots of the Mexican barbasco plant can serve as the starting material.

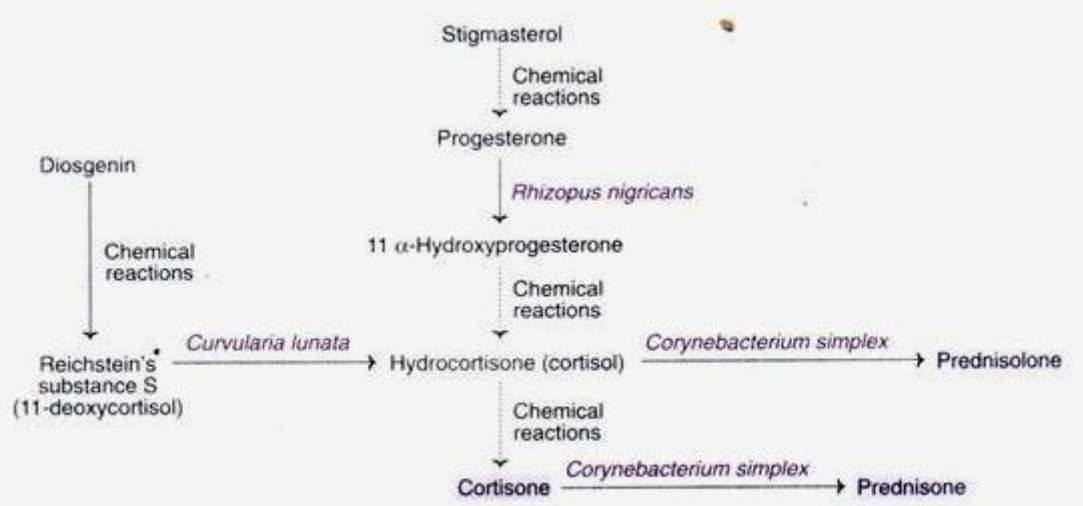


Fig. 22.1 : Biotransformation of commercially important steroids.

Stigma sterol can be chemically converted to progesterone which is subjected to bio-transformation to form 11 α-hydroxyprogesterone by the microorganism, Rhizopus nigricans. Cortisol (hydrocortisone), produced from 11 α-hydroxyprogesterone by chemical reactions, undergoes microbial transformation (organism-Corynebacterium simplex) to form prednisolone.

Further, cortisone formed from Cortisol can be subjected to biotransformation by Corynebacterium simplex to produce prednisone. When diosgenin is used as the starting compound, substance S can be produced by chemical reactions which can be converted to Cortisol by biotransformation with the help of the microorganism Curvularia lunata.

Biotransformation of steroids is usually carried out by batch fermentation. Use of immobilized cells or immobilized enzymes is gaining importance in recent years. This is advantageous since the biotransformation is more efficient with high substrate concentration, short conversion time and good product recovery.

Since the steroids are not water soluble, the microbial transformation reactions have to be carried out in organic solvent (water-immiscible) system. However, the organic solvents are toxic to micro-organisms or enzymes. It is ideal to use an aqueous two phase system for biotransformation of steroids.

Biotransformation of cholesterol:

Certain commercially important steroids (e.g. androstendione, androstadiendione) can be produced directly from cholesterol by biotransformation (Fig. 22.2).

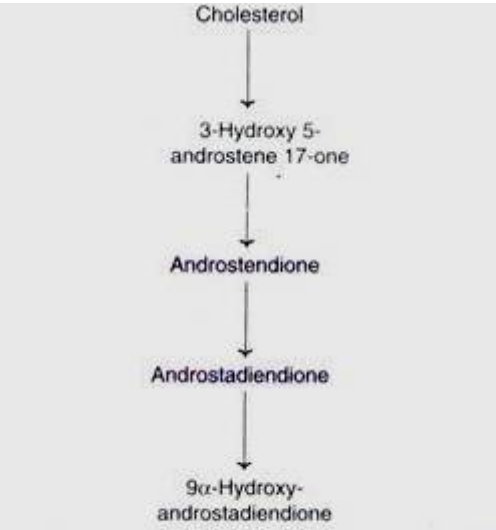


***Fig. 22.2** : Biotransformation of cholesterol by mycobacteria to commercial products.*

2. Biotransformation of Antibiotics:

Production of new antibiotics or modifications in the existing ones for more effective treatment of the diseases is always on the priority of the pharmaceutical industry. Further, antibiotics with wider antimicrobial spectrum, reduced toxicity, low allergic reactions and decreased resistance are highly advantageous. Biotransformation reactions significantly contribute for improving the pharmaceutical products.

Direct biotransformation:

Acylation and de-acylation, phosphorylation, adenylation and hydrolysis are some of the reactions involved in the microbial transformation of antibiotics.

Biotransformation of penicillin G:

Microbial transformation, in association with chemical synthesis, is routinely used for the commercial production of semisynthetic penicillin's and cephalosporin's. The enzymatic cleavage of penicillin by penicillin acylase into 6-amino- penicillanic acids is a very important reaction (Fig. 22.3). Penicillin G gets inactivated by its conversion to benzylpenicilloic acid by the enzyme penicillinase (β-lactamase).

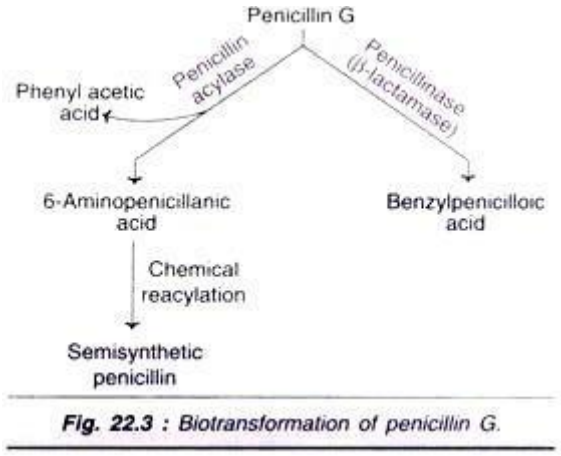


Fig. 22.3 : Biotransformation of penicillin G.

Biotransformation of narbomycin:

Hydroxylation of narbomycin to picromycin (brought out by Streptomyces sp) is another good example of microbial transformation.

Biotransformation of macrolides:

The macrolide antibiotics on de-acylation will give less active products. These products can be used for the production of more active semisynthetic macrolides.

Indirect biotransformation:

The biosynthetic processes of antibiotics can be controlled by the addition certain inhibitors or modified substrates to the medium. In other words, the biosynthesis of antibiotics occurs in a controlled fashion in the indirect biotransformation.

Biotransformation of actinomycins:

The microorganism Streptomyces parvulus produces new actinomycins in the presence of 4-methyl- proline (proline analog) in the medium. The new antibiotics will have 4-methylproline in place of proline and these actinomycins are more efficient in their function.

Biotransformation of ribostamycin:

In the biosynthesis of neomycin, ribostamycin is an intermediate. By employing mutant strains of Streptomyces fradiae, ribostamycin can be produced in large quantities. Several other mutant strains of microorganisms have been created by recombinant DNA technology for the production of modified antibiotics of aminoglycosides and rifamycins.

3. Biotransformation of Arachidonic Acid to Prostaglandins:

Prostaglandins (PG) have a wide spectrum of biological functions. They are important for pharmaceutical and therapeutic purposes. For instance, PGE_1 serves as a contraceptive; PGG_1 is used in the treatment of congenital heart failure; PGG_2 for relieving labour pains.

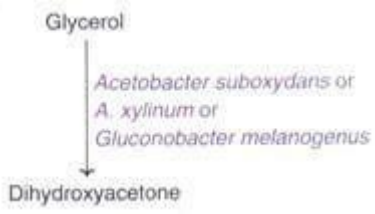
The unsaturated fatty acid arachidonic acid is the precursor for the biosynthesis of prostaglandins. Some success has been reported in the biotransformation of arachidonic acid to PGE_1, PGE_2, PGF_1 and PGF_2 by using fungi. It is expected that in the coming years, prostaglandins with improved efficiency will be produced by bio-transformations.

4. Biotransformation for the Production of Ascorbic Acid:

Ascorbic acid (vitamin C) can be commercially produced by a combination of chemical and microbial transformation processes.

5. Biotransformation of Glycerol to Dihydroxyacetone:

Dihydroxyacetone is used in cosmetics and suntan lotions. Certain acetic acid bacteria can convert glycerol to dihydroxyacetone through the process of biotransformation.



Good oxygen supply, temperature 26-28°C and pH 6.0 are ideal for the optimal biotransformation.

6. Biotransformation for the Production of Indigo:

Indigo can be synthesized by microbial transformation. This has been made possible by cloning a single Pseudomonas gene that encodes naphthalene di-oxygenase in the creation of E. coli. The relevant reactions of biotransformation for the production of indigo are depicted in Fig. 22.4.

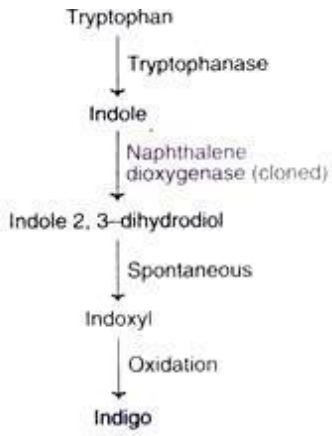


Fig. 22.4 : Microbial production of indigo.

UNIT III

Degradation of pollutants

Meaning of Xenobiotic Compounds:

Xenobiotic compounds are man-made chemicals that are present in the environment at unnaturally high concentrations. The xenobiotic compounds are either not produced naturally, or are produced at much lower concentrations than man. Microorganism have the capability of degrading all naturally occurring compounds; this is known as the principle of microbial infallibility proposed by Alexander in 1965.

Microorganisms are also able to degrade many of the xenobiotic compounds, but they are unable to degrade many others. The compounds that resist biodegradation and thereby persists in the environment are called recalcitrant.

The xenobiotic compounds may be recalcitrant due to one or more of the following reasons:

(i) They are not recognised as substrate by the existing degradative enzymes,

(ii) They are highly stable, i.e., chemically and biologically inert due to the presence of substitution groups like halogens, nitro-, sulphonate, amino-, methoxy- and carbamyl groups,

(iii) They are insoluble in water, or are adsorbed to external matrices like soil,

(iv) They are highly foxic or give rise to toxic products due to microbial activity,

(v) Their large molecular size prevents entry into microbial cells,

(vi) Inability of the compounds to induce the synthesis of degrading enzymes, and

(vii) Jack of the perm-ease needed for their transport into the microbial cells.

Types of Recalcitrant Xenobiotic Compounds:

The recalcitrant xenobiotic compounds can be grouped into the following 6 types:

(i) Halocarbons,

(ii) Polychlorinated biphenyls,

(iii) Synthetic polymers,

(iv) Alkylbenzyl sulphonates,

(v) Oil mixture and

(vi) Others.

The structural features that make these compounds resistant to microbial degradation include the following:

(i) Presence of halogens in the place of hydrogen in the molecule; the carbon-halogen bond is highly stable and its cleavage requires considerable energy,

(ii) Substitution of H by other groups like nitro-, sulphonate, methoxy-, amino- and carbomyl groups,

(iii) Cyclic structures, aromatic compounds, cycloalkanes and heterocyclic compounds are more recalcitrant than linear chain or aliphatic compounds,

(iv) Branched linear chains resist biodegradation etc.

In general, the more complex is the structure of a xenobiotic compound, the more resistant it is to biodegradation. Many other xenobiotics resist biodegradation due to their large molecular size and insolubility in water.

(i) Halocarbons:

These compounds contain different numbers of halogen (e.g., CI, Br, F (fluorine), I) atoms in the place of H atoms. They are used as solvents (chloroform, $CHCI_3$), as propellants in spray cans of cosmetics, paints etc., in condenser units of cooling systems (Freons, CCI_3F, CCl_2F_2, $CClF_3$, CF_4), and as insecticides (DDT, BHC, lindane etc.) and herbicides (dalapon, 2, 4-D, 2, 4, 5-T etc.).

The C_1-C_2 haloalkanes like chloroform, freons etc. are volatile and escape into the atmosphere where they destroy the protective ozone (O_3) layer leading to increased UV radiation. Pesticides (herbicides, fungicides and insecticides) are applied to crops from where they leach into water bodies; many of them are subject to bio-magnification.

(ii) Poly chlorinated Biphenyls (PCB's):

These compounds have two covalently linked benzene rings having halogens substituting for H. PCB's are used as plasticisers, insulator coolants in transformers and as heat exchange fluids. They are both biologically and chemically inert to various degrees, which increases with the number of chlorine atoms present in the molecule.

The recalcitrant nature of the above two groups of compounds is due to their halogenation and as well their cyclic structure (PCB's).

(iii) Synthetic Polymers:

These compounds are produced as plastics, e.g., polyethylene, polystyrene, polyvinyl chloride etc., and nylons which are used as garments, wrapping materials etc. They are recalcitrant mainly due to their insolubility in water and molecular size.

(iv) Alkylbenzyl Sulfonates:

These are surface-active detergents superior to soaps. The sulphonate (— SO_3^-) group present at one end resists microbial degradation, while the other end (non-polar alkyl end) becomes recalcitrant if its is branched, (resistance increases with the degree of branching). At present, alkylbenzyl sulphonates having non-branched alkyl ends are used; these are biodegraded by β-oxidation from their alkyl ends.

(v) Oil Mixtures:

Oil is a natural product, has many components and is biodegradable, the different components being degraded at different rates. Biodegradation is able to handle small oil seepages. But when large spills occur the problem of pollution becomes acute. Oil is recalcitrant mainly because of its insolubility in water and due to the toxicity of some of its components.

(vi) Other Xenobiotic Compounds:

A number of pesticides are based on aliphatic, cyclic ring structures containing substitution of nitro-, sulphonate, methoxy-, amino- and carbomyl groups; in addition, they also contain halogens. These substitutions make them recalcitrant.

Hazards from Xenobiotic Compounds:

The xenobiotics present a number of potential hazards to man and the environment which are briefly listed below.

(i) Toxicity:

Many xenobiotics like halogenated and aromatic hycrocarbons are toxic to bacteria, lower eukaryotes and even humans. At low concentrations they may cause various skin problems and reduce reproductive potential.

(ii) Carcinogenicity:

Certain halogenated hydrocarbons have been shown to be carcinogenic.

(iii) Many xenobiotics are recalcitrant and persist in the environment so that there is a build up in their concentration with time.

(iv) Many xenobiotics including DDT and PCB's are recalcitrant and lipophilic; as a consequence they show bioaccumulation or bio-magnification often by a factor of $10^4 – 10^6$.

Bio-magnification occurs mainly because of the following two reasons:

(i) These compounds are continuously taken up from the environment and accumulated in the lipid deposits of body, e.g., a 100-fold accumulation of DDT by plankton from water,

(ii) Such organisms are consumed by other organisms in a sequential manner constituting the food chain, e.g., plankton → small fish → large fish → sea-eagles; the concentration of xenobiotics builds up as we move up in the food chain (Table 32.1).

In case of DDT a 10^5 – fold increase occurs in sea-eagles as compared to the concentration present in the aqueous environment as a result of which sea-eagles laid fragile eggs. DDT and PCB's have been found in human tissues in high but sub-lethal concentrations in those countries where they have been used, although humans were often not in direct contact with these chemicals.

TABLE 31.1. Biomagnification of DDT by continued absorption by plankton and the subsequent passage of planktons and fishes through the food chain (plakton → small fish → large fish → sea-eagle)

Organism environment	*DDT concentration*	*fold increase over concentration in water*
Water	0.3 ppb*	—
Plankton	30 ppb	100 (= 10^2)
Small fish	0.3 ppm**	1,000 (= 10^3)
Large fish	3 ppm	10,000 (= 10^4)
Sea-eagle	30 ppm	100,000 (= 10^5)

*ppb = parts per billion (1 billion = 1000 million)
**ppm = parts per million (1 million = 1,00,000)

(v) They are produced and used in large quantities which favours their accumulation in nature.

General Features of Biodegradation of Xenobiotics:

Since xenobiotics consist of a wide variety of compounds, their degradation occurs via a large number of metabolic pathways.

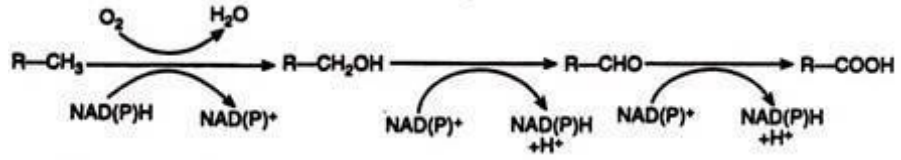
Degradation of alkanes and aromatic hydrocarbons generally occurs as follows:

(i) An oxygenase first introduces a hydroxyl group to make the compound reactive,

(ii) The hydroxyl group is then oxidised to a carboxyl group,

(iii) The ring structure is opened up (in case of cyclic compounds),

(iv) The linear molecule is degraded by β-oxidation to yield acetyl CoA which is metabolised in the usual manner. For example, an n-alkane is oxidised as follows.



Similarly, an alicyclic hydrocarbon, e.g., cyclohexane, is oxidised as follows:

(i) First an oxygenase adds an —OH group in the ring,

(ii) Then another oxygenase forms an ester in the form of a lactone,

(iii) Which is then hydrolysed to open the ring structure to yield a linear molecule (Fig. 32.12).

In both these oxidations mono-oxygenases are involved which add oxygen to a single position in the molecule. In contrast, oxidation of benzene ring may involve a di-oxygenase which adds oxygen at two positions in the molecule in a single step (Fig. 32.13).

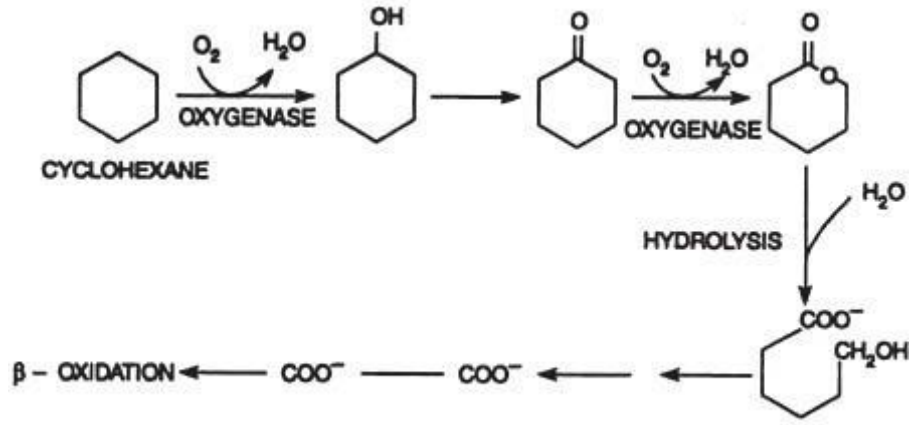


FIG. 32.12. Oxidation of cyclohexane. Two different mono-oxygenases are involved.

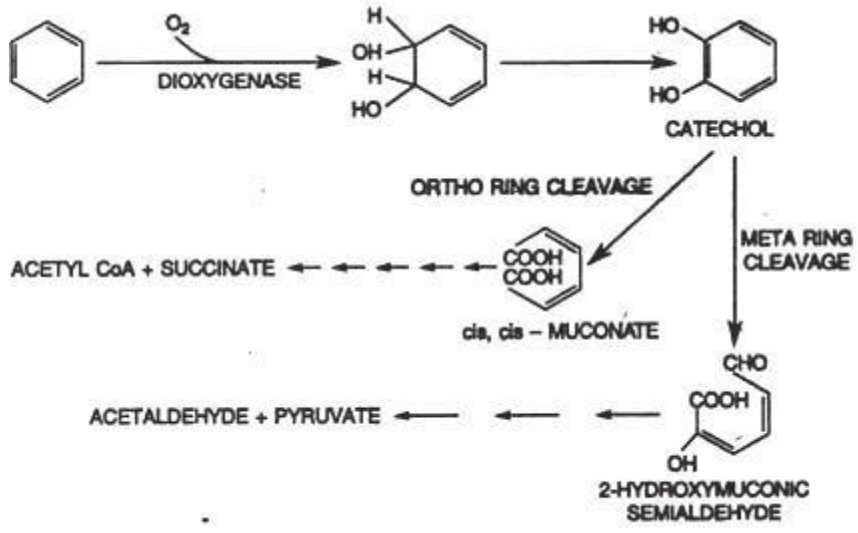


FIG. 32.13. Oxidation of aromatic hydrocarbons initiated by the action of a di-oxygenase.

Both mono- and di-oxygenases are of a variety of types: some react best with short chain alkanes, while others act on cyclic alkanes. But these enzymes are not very specific and each enzyme oxidise a limited range of compounds. Thus xenobiotics are degraded by a wide variety of microorganisms, each of which degrades a small range of compounds.

Frequently, oxidation of xenobiotics involves cytochrome P_{450} or rebredoxin. In addition, the halogens and/or other substituent groups are either modified or removed usually as one of the initial reactions or sometimes it is achieved later in the process.

Hydrocarbon Degradation:

The degradation of hydrocarbons is briefly outline below:

(i) Halomethanes:

Halomethanes are transferred into methanol by the enzyme methane mono-oxygenase which uses them as substrate; this enzyme occurs in a number of methylotrophs. Alternatively, a glutathione-dependent hydrolase catalyses oxidative dechlorination of halomethanes into methanol; this reaction is anaerobic and uses oxygen derived from water. Methanol is oxidised to $CO_2 + H_2O$ via formaldehyde and formic acid.

(ii) Cyanide:

Cyanide(HCN) is toxic to biological systems and even microorganisms capable of degrading cyanide cannot withstand a high concentration of HCN. Some of the cyanides, e.g., HCN and CH_3CN, are volatile. Therefore, disposal of cyanide is strictly controlled.

It is degraded as follows:

$$HCN \xrightarrow{\text{Hydratase (fungal)}} HCONH_2$$

$$HCN \xrightarrow[\textit{Pseudomonas fluorescens}]{\text{Bacteria}} CO_2 + NH_2$$

(iii) Aliphatic Hydrocarbons:

Aliphatic Hydrocarbons may be saturated or unsaturated, n-Alkanes of 10-24 carbons are the most readily biodegraded. Similarly saturated aliphatics are easier to degrade than unsaturated ones and branched chains show decreased biodegradation. Biodegradation of n-alkanes is catalysed by oxygenases to produce carboxylic acid, which is then degraded by β-oxidation.

Oxidation may involve the methane group at one end of n-alkane molecule, or it may occur at a β-methylene group (Fig. 32.14). Sometimes, both terminal methyl groups are oxidised to

yield a dicarboxylic acid; this reaction is used by many microorganisms for the biodegradation of branched chain n-alkanes.

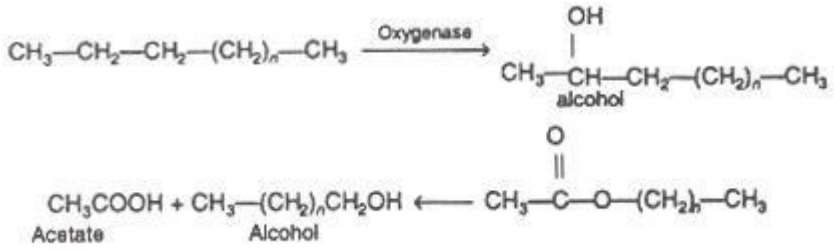


FIG. 32.14. Oxidation of a *b*-methylene group of an *n*-alkane. The resulting acetate molecule is further oxidised as usual

(iv) Alicyclic Hydrocarbons:

Alicyclic hydrocarbons are present naturally in waxes from plants, crude oil, microbial lipids etc. and are represented by xenobiotics used as pesticides and also in petroleum products. Un-substituted cyclohexane rings are oxidised as outlines in Fig. 32.12.

(v) Aromatic Hydrocarbons:

Aromatic hydrocarbons are rather stable.

These are oxidised by di-oxygenascs (Fig. 32.13) to catechol which is further metabolised by two separate pathways:

(i) In case of ortho-ring cleavage pathway, a 1, 2- dioxygenase cleaves the ring between the two adjacent hydroxyl groups and sequential catabolism of the product cis, cis-muconate yields succinate + acetyl CoA (Fig. 32.13).

(ii) Alternatively, the enzyme 2, 3-di- oxygenase cleaves the ring between the carbon atom having an OH group and an adjacent carbon lacking an OH group (meta-cleavage); the products at the end of reaction sequence are acetaldehyde and pyruvate. Both ortho and meta pathways arc involved in degradation of aromatic hydrocarbons. Benzene is degraded by the meta pathway.

(vi) Polycyclic Hydrocarbons:

Polycyclic hydrocarbons contain two or more rings. Generally, one of the terminal rings is attacked by a di-oxygenase, leading to ring cleavage and degradation so that in the end a single ring remains which is catabolised in a manner similar to that described above (Fig. 32.13).

Degradation of complex molecules containing aliphatic, aromatic, alicyclic or heterocyclic components is difficult to generalise but the following features are observed:

(i) Amide-, ester- or ether bonds are first attacked and further degradation of the products so generated takes place;

(ii) If these bonds are absent or inaccessible; aliphatic chains are degraded;

(iii) If aliphatic chains are branched, the aromatic component of complex molecules may be attacked,

(iv) The site and mode of attack depends on the molecular structure, the microorganism involved and the environmental conditions,

(v) In general, recalcitrance of various benzene derivatives increases with the substituent groups (at meta position) as follows: COOH = OH < $-NH_2$ < $-O—CH_3$ < $-SO_3^-$ < $-NO_2^-$. Further, the greater the number of substituent groups on the benzene ring, the higher the degree of recalcitrance. (ii) The position of substitution also affects recalcitrance as meta > ortho > para in recalcitrance (Fig. 32.15).

FIG. 32.15. The relative effects of the presence of a given substituent group at different positions in a benzene ring.

Co-Metabolism and Gratuitous Metabolism of Xenobiotic Compounds:

Some xenobiotic compounds, e.g., cyclohexane, halogenated compounds etc., are degraded by microbes, but these compounds are rarely, if ever, used as sources of energy and carbon by them.

Degradation of such compounds, therefore, depends on the presence of another compound which induces the necessary enzymes, and metabolism of which provides both energy and reducing equivalents for the degradation of xenobiotic compounds (and the C, energy etc. needed for microbial growth).

Clearly in such cases, degradation of xenobiotic compounds depends on the presence and metabolism of a suitable substrate called co-metabolite, such a degradation is called co-metabolism.

In contrast, several xenobiotic compounds are degraded by an existing pathway and are used by microbes as sources of energy and reducing equivalents; this is known as gratuitous metabolism. In such a metabolism, the necessary enzymes are already induced by another compound which is not needed as a co-metabolite.

The xenobiotics degraded by both gratuitous and co-metabolism are very similar to the natural substrates of the enzymes involved in their degradation. Often a xenobiotic compound may not be completely degraded by gratuitous metabolism, but the product may be less polluting or may be used as substrate by some organisms.

Biodegradation of Halogenated Compounds:

Biodegradation of such compounds involves two distinct steps:

(i) Elimination of the halogen groups, and

(ii) Degradation of the non-halogenated product molecule.

Removal of halogen molecule may occur either directly involving the removal of hydrogen halide (e.g., HCI, Fig. 32.16), or it may involve the substitution of halogen by —H (Fig. 32.16B), -OH (Fig. 32.16C) or a -thio group (Fig. 32.16D).

The direct halogen removal produces a double bond and is relatively rate in nature. The mechanism involving halogen substitution, especially by —OH, is far more common particularly for fully reduced aliphatics or aromatics.

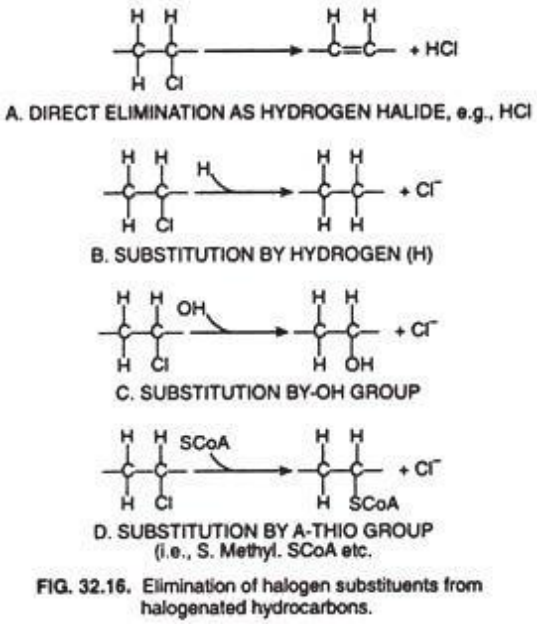


FIG. 32.16. Elimination of halogen substituents from halogenated hydrocarbons.

The aerobic degradation of halogenated aromatic compounds usually involves the following steps:

(i) Addition of —OH group by a dioxygenase to yield chlorinated catechol's (as in case of normal benzene; Fig. 32.13),

(ii) Cleavage of the ring by ortlio or meta cleavage (meta cleavage often results in the halogen not being eliminated from the aliphatic product which may lead to an accumulation of toxic metabolites),

(iii) Elimination of the halogen from the straight chain (alophatic) product, and finally,

(iv) Degradation of the aliphatic hydrocarbon (non-halogenated) so produced. In case of phenols (which already have one —OH group), the step 1 reaction is catalysed by a hydroxylase which adds another —OH group to yield the catechols.

Rarely, however, the halogen may be removed before cleavage of the aromatic ring. Usually, the removal of halogen' occurs by its substitution with a —H or —OH group; the resulting non-halogenated aromatic compound is then metabolized as depicted in Fig. 32.13.

The Origin of Capacity to Degrade Xenobiotics:

Continued exposure of microorganisms to xenobiotic compounds can often lead to the evolution of metabolic processes needed to wholly or partly degrade the xenobiotic.

These capabilities may arise due to:

(i) Mutation, and

(ii) Transfer of plasmid borne genes.

Gene mutation occurs spontaneously at a low frequency. But the short generation time and very large populations of microorganisms make mutation a potent source of genetic variation. Mutations can be expected to either modify the active site of an enzyme so that it has an increased affinity for the xenobiotic, or it can eliminate regulatory controls and enhance its production. Such changes only enhance the rate of degradation of a xenobiotic; they rarely, if ever, generate a new enzyme function.

Often new enzyme activities are acquired by plasmid transfer (usually through conjugation) since many of the key enzymes concerned with xenobiotic metabolism are plasmid borne. Most bacteria containing such plasmids are gram-negative aerobes mainly from the genes Pseudomonas. Some plasmids encode the entire pathway for xenobiotic degradation, e.g., TOL plasmids encode the enzymes for toluene degradation.

But many plasmids encode only some of the degradative enzymes, e.g., pAC21 for p-chlorobiphenyl degradation and pAC25 for 3-chlorobenzoate degradation; in such cases, the remaining enzymes involved in the degradation must be provided either by the chromosomal genes of the cell or by another microorganism.

Plasmid transfers allow a microorganism:

(i) To acquire the genes needed to complete the pathway for a xenobiotic metabolism, and/or

(ii) To gain genes which improves the rate and/or the nature of degradation.

The ability of plasmid transfer can be exploited to create microorganisms with novel characteristics. For example, Alcaligenes sp. degrade 4-chlorophenol to 5-chloro-2-hydroxymuconic semialdehyde (by meta cleavage of the ring) which is toxic.

When the concentration of 4-chlorophenol is higher than 4 m mol/1, the level of this intermediate becomes toxic preventing its further degradation. In contrast Pseudomonas strain B13 has a plasmid-borne gene which encodes the enzyme 1, 2-di-oxygenase; this enzyme cleaves 4-chlorophenol by ortho pathway which avoids the production of toxic intermediates (Fig. 32.17).

When a mixture of Alcaligenes sp. and Pseudomonas strain B13 were maintained in laboratory, the Alcaligenes sp. acquired the plasmid and the ability for ortho ring cleavage from Pseudomonas strain B13. This is an example of the production of a natural recombinant species of bacteria. Attempts are being made to develop genetically engineered bacteria having recombinant pathways for xenobiotic compound degradation.

The strategies most likely to succeed are:

(i) To transfer genes encoding enzymes with a wider specificity for substrates, and

(ii) To modify regulatory elements with a view to enhance enzyme synthesis, to promote assimilation of the xenobiotic compound into the cell etc.

The possibilities of creating a ‘super bug’ capable of degrading a wide variety of xenobiotic compounds under a number of conditions in stable association with native microorganisms seems unrealistic.

This is because microorganisms carrying additional recombinant DNA are poor competitors against native microorganisms. In addition, the release of genetically engineered microbes (GEMs) in the environment is illegal in many countries.

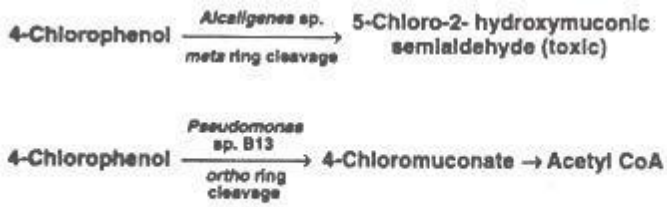


FIG. 32.17. The consequences of *meta* and ortho pathways of metabolism of 4-chorophenol by *Alcaligenes* sp. and *Pseudomonas* sp. B13, respectively.

Use of Mixed Microbial Populations:

The use of mixed populations of microbes for degradation of xenobiotic compounds is desirable due to the following reasons:

(i) Two different microbes can together degrade degrade a xenobiotic completely, while either of them alone is incapable of this feat. In such a case, the product of degradation by one microorganism serves as the substrate for the other.

For example, Acinetobacter sp. has plasmid-borne genes for dihydroxylation of one of the rings of 4-chlorobiphenyl, its meta ring cleavage and subsequent degradation to produce 4-chlorobenzoate; however, it can not degrade this product any further.

A Pseudomonas putida strain cleaves the ring of 4-chlorobenzoate by ortho pathway, to ultimately generate acetyl-CoA and succinate; but this bacterium can not utilize 4-chlorobiphenyl.' Thus Acinetobacter and P. putida act in tandem to degrade completely the xenobiotic 4-chlorobiphenyl which neither of them can accomplish on its own (Fig. 32.18).

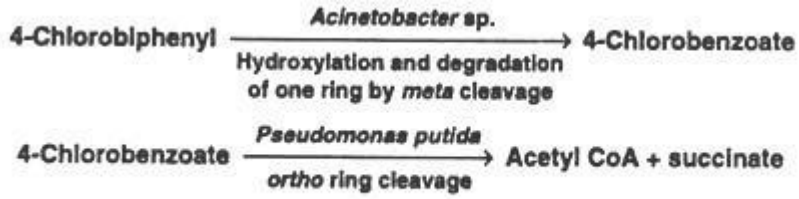


FIG. 32.18. Joint tandem action of *Acinetobacter* and *Pseudomonas putida* in degradation of 4-chlorobiphenyl.

(ii) One microorganism may produce growth factor/nutrient required by the other. For example, Nocardia sp. degrades cyclohexane but is unable to produce biotin. A Pseudomonas sp. strain produces biotin but can not degrade cyclohexane. Nocardia sp. breakdown cyclohexane; the breakdown products and Nocardia cell lysis products are used by Pseudomonas strain which grows and releases biotin.

The biotin, in turn, promotes Nocardia growth and cyclohexane breakdown. Thus a mixture of these two strains would breakdown cyclohexane but neither of them can do it alone.

(iii) Co-culture may lead to plasmid transfer into a faster growing species thereby creating a faster growing species capable of degrading the xenobiotic. An example is the transfer of plasmid from Pseudomonas sp. strain B13 into Alcaligenes sp. which is faster growing than Pseudomonas sp. B13.

(iv) In natural environments, mixtures of xenobiotic compounds are found. Use of mixed cultures increases the likelihood of the microbial components of mixed culture being able to degrade all the xenobiotic compounds present.

(v) The biological treatment system, i.e., the microbial community used for degrading xenobiotics, is more stable and can withstand occasional shock loadings.

(vi) Biodegradation rates are usually higher due to the microbial interactions described above.

Practical Approaches to Xenobiotic Degradation:

Biodegradation of xenobiotic compounds depends on their concentration (too high concentration may be toxic), pH of the medium, temperature, availability of water and other nutrients and presence of organic compounds (these may be co-metabolites, inhibitors or preferred substrate, in place of the xenobiotic, by microorganisms). In general, the xenobiotic compound should be available in an acceptable concentration and toxic levels should not occur.

In a treatment system, a constant supply of the compound should be available for selective maintenance of microbes capable of its degradation. In addition, interfering organic compounds should not be present in the environment.

Practical application of microbes for xenobiotic degradation is facilitated by:

(i) Supply of sufficient nutrients or co-metabolites,

(ii) Maintenance of the xenobiotic compounds to non-toxic levels and

(iii) Provision of microbial population or inoculum.

(i) Nutrients and Co-metabolites:

All the nutrients required for growth and metabolism of microbial cells must be available in the solution containing the xenobiotic compound. In general, nitrogen,- phosphorus and, sometimes, sulphur may have to be added to the solution.

Where necessary, adequate 0_2 supply should be ensured by aeration of the medium since xenobiotic compounds are degraded aerobically. In addition, some compounds may be

required as co-metabolites to induce the necessary enzymes and to provide the energy and the reducing equivalents for xenobiotic degradation.

(ii) Xenobiotic Concentration:

In case the xenobiotic concentration is high, it should be reduced by appropriate dilution with, usually, water. In continuous treatment system, e.g., for effluents, the amount of xenobiotic entering the system should not exceed the amount being degraded by the system. At the same time, xenobiotic should always to present in the effluent in order to maintain the necessary microbial population. However, when the concentration of the xenobiotic compound is relatively low, a higher density of the degradative microbes will be needed for degradation of the compound.

(iii) Microbial Inoculum:

In a treatment system natural evolution of the microbial population will take time. Therefore, it is customary to inoculate the system with a suitable population of microbes having the necessary degradative capabilities; this is called bio-augmentation. A number of inoculants designed to degrade various xenobiotic compounds are commercially available.

These inoculants consist of naturally occurring microbial species which have been isolated, purified and characterised for various features, including their xenobiotic degradation capabilities. The stocks of various isolates are preserved by air-drying or freeze-drying techniques.

The commercial inoculum is prepared by selecting the strains needed for degradation of the specified xenobiotic compounds, and then matching and blending them; bulking agents, dispersant chemicals, wetting agents and nutrients are also added. The inoculants are usually available as dry powders which are easy to store, transport and reconstitute at site (by simply adding water).

BIODEGRADATION AND BIOREMEDIATION AND ITS TYPES

Biodegradation or biological degradation is the phenomenon of biological transformation of organic compounds by living organisms, particularly the microorganisms.

Biodegradation basically involves the conversion of complex organic molecules to simpler (and mostly non-toxic) ones. The term biotransformation is used for incomplete biodegradation of organic compounds involving one or a few reactions. Biotransformation is employed for the synthesis of commercially important products by microorganisms.

Bioremediation refers to the process of using microorganisms to remove the environmental pollutants i.e. the toxic wastes found in soil, water, air etc. The microbes serve as scavengers in bioremediation. The removal of organic wastes by microbes for environmental clean-up is the essence of bioremediation. The other names used (by some authors) for bioremediation are bio-treatment, bio-reclamation and bio-restoration.

It is rather difficult to show any distinction between biodegradation and bioremediation. Further, in biotechnology, most of the reactions of biodegradation/bioremediation involve xenobiotic.

Xenobiotic:

Xenobiotic (xenos-foregin) broadly refer to the unnatural, foreign and synthetic chemicals such as pesticides, herbicides, refrigerants, solvents and other organic compounds. Microbial degradation of xenobiotic assumes significance, since it provides an effective and economic means of disposing of toxic chemicals, particularly the environmental pollutants.

Pseudomonas — The Predominant Microorganism For Bioremediation:

Members of the genus Pseudomonas (a soil microorganism) are the most predominant microorganisms that degrade xenobiotic. Different strains of Pseudomonas, that are capable of detoxifying more than 100 organic compounds, have been identified. The examples of organic compounds are several hydrocarbons, phenols, organophosphates, polychlorinated biphenyls (PCBs) and polycylic aromatics and naphthalene.

About 40-50 microbial strains of microorganisms, capable of degrading xenobiotics have been isolated. Besides Pseudomonas, other good examples are Mycobacterium, Alcaligenes,

and Nocardia. A selected list of microorganisms and the xenobiotics degraded is given in Table 59.1.

Table 59.1 A selected list of microorganisms and the pollutants (xenobiotics) that are degraded by bioremediation

Microorganism	*Pollutant chemicals*
Pseudomonas sp	Aliphatic and aromatic hydrocarbons—alkylaminoxides, alkylammonium benzene, naphthalene, anthracene xylene, toluene, polychlorinated biphenyls (PCBs), malathion, parathion, organophosphates.
Mycobacterium sp	Benzene, branched hydrocarbons, cycloparaffins
Alcaligenes sp	Polychlorinated biphenyls, alkyl benzene, halogenated hydrocarbons.
Nocardia sp	Naphthalene, alkylbenzenes, phenoxyacetate.
Arthrobacter sp	Benzene, polycyclic aromatics, phenoxyacetate, pentachlorophenol.
Corynebacterium sp	Halogenated hydrocarbons, phenoxyacetate.
Bacillus sp	Long chain alkanes, phenylurea.
Candida sp	Polychlorinated biphenyls
Aspergillus sp	Phenols
Xanthomonas sp	Polycyclic hydrocarbons
Streptomyces sp	Halogenated hydrocarbons, phenoxyacetate.
Fusarium sp	Propanil
Cunninghamella sp	Polycyclic aromatics, polychlorinated biphenyls.

Consortia of microorganisms for biodegradation:

A particular strain of microorganism may degrade one or more compounds. Sometimes, for the degradation of a single compound, the synergetic action of a few microorganisms (i.e. a consortium or cocktail of microbes) may be more efficient. For instance, the insecticide parathion is more efficiently degraded by the combined action of Pseudomonas aeruginosa and Psudomonas stulzeri.

Co-metabolism in biodegradation:

In general, the metabolism (breakdown) of xenobiotics is not associated with any advantage to the microorganism. That is the pollutant chemical cannot serve as a source of carbon or energy for the organism. The term co-metabolism is often used to indicate the non-beneficial (to the microorganism) biochemical pathways concerned with the biodegradation of xenobiotics. However, co- metabolism depends on the presence of a suitable substrate for the microorganism. Such compounds are referred to co-substrates.

Factors Affecting Biodegradation:

Several factors influence biodegradation. These include the chemical nature of the xenobiotic, the capability of the individual microorganism, nutrient and O_2 supply, temperature, pH and redox potential. Among these, the chemical nature of the substrate that has to be degraded is very important.

Some of the relevant features are given hereunder:

i. In general, aliphatic compounds are more easily degraded than aromatic ones.

ii. Presence of cyclic ring structures and length chains or branches decrease the efficiency of biodegradation.

iii. Water soluble compounds are more easily degraded.

iv. Molecular orientation of aromatic compounds influences biodegradation i.e. ortho > para > meta.

v. The presence of halogens (in aromatic compounds) inhibits biodegradation.

Besides the factors listed above, there are two recent developments to enhance the biodegradation by microorganisms.

Bio-stimulation:

This is a process by which the microbial activity can be enhanced by increased supply of nutrients or by addition of certain stimulating agents (electron acceptors, surfactants).

Bio-augmentation:

It is possible to increase biodegradation through manipulation of genes. More details on this genetic manipulation i.e. genetically engineered microorganisms (GEMs), are described later. Bio-augmentation can also be achieved by employing a consortium of microorganisms.

Enzyme Systems for Biodegradation:

Several enzyme systems (with independent enzymes that work together) are in existence in the microorganisms for the degradation of xenobiotics. The genes coding for the enzymes of bio-degradative pathways may be present in the chromosomal DNA or more frequently on the plasmids. In certain microorganisms, the genes of both chromosome and plasmid contribute for the enzymes of biodegradation. The microorganism Pseudomonas occupies a special place in biodegradation.

A selected list of xenobiotics and the plasmids containing the genes for their degradation is given in Table 59.2.

TABLE 59.2 A selected list of xenobiotics and the plasmids containing genes (in *Plasmodium*) for biodegradation

Xenobiotic	*Name of plasmid in* Pseudomonas
Naphthalene	NAH
Xylene	XYL
Xylene and toluene	TOL, pWWO, XYL-K
Salicylate	SAL
Camphor	CAM
3-Chlorobenzene	pAC25

Recalcitrant Xenobiotics:

There are certain compounds that do not easily undergo biodegradation and therefore persist in the environment for a long period (sometimes in years). They are labeled as recalcitrant.

There may be several reasons for the resistance of xenobiotics to microbial degradation:

i. They may chemically and biologically inert (highly stable).

ii. Lack of enzyme system in the microorganisms for biodegradation.

iii. They cannot enter the microorganisms being large molecules or lack of transport systems.

iv. The compounds may be highly toxic or result in the formation highly toxic products that kill microorganisms.

There are a large number of racalcitrant xenobiotic compounds e.g. chloroform, freons, insecticides (DDT, lindane), herbicides (dalapon) and synthetic polymers (plastics e.g. polystyrene, polyethylene, polyvinyl chlorine).

It takes about 4-5 years for the degradation of DDT (75-100%) in the soil. A group of microorganisms (Aspergillus flavus, Mucor aternans, Fusarium oxysporum and Trichoderma viride) are associated with the slow biodegradation of DDT.

Bio-magnification:

The phenomenon of progressive increase in the concentration of a xenobiotic compound, as the substance is passed through the food chain is referred to as bio-magnification or bioaccumulation. For instance, the insecticide DDT is absorbed repeatedly by plants and microorganism.

When they are eaten by fish and birds, this pesticide being recalcitrant, accumulates, and enters the food chain. Thus, DDT may find its entry into various animals, including man. DDT affects the nervous systems, and it has been banned in some countries.

Types of Bioremediation:

The most important aspect of environmental biotechnology is the effective management of hazardous and toxic pollutants (xenobiotics) by bioremediation. The environmental clean-up process through bioremediation can be achieved in two ways—in situ and ex situ bioremediation.

In Situ Bioremediation:

In situ bioremediation involves a direct approach for the microbial degradation of xenobiotics at the sites of pollution (soil, ground water). Addition of adequate quantities of nutrients at the sites promotes microbial growth. When these microorganisms are exposed to xenobiotics (pollutants), they develop metabolic ability to degrade them.

The growth of the microorganisms and their ability to bring out biodegradation are dependent on the supply of essential nutrients (nitrogen, phosphorus etc.). In situ bioremediation has been successfully applied for clean-up of oil spillages, beaches etc. There are two types of in situ bioremediation-intrinsic and engineered.

Intrinsic bioremediation:

The inherent metabolic ability of the microorganisms to degrade certain pollutants is the intrinsic bioremediation. In fact, the microorganisms can be tested in the laboratory for their natural capability of biodegradation and appropriately utilized.

Engineered in situ bioremediation:

The inherent ability of the microorganisms for bioremediation is generally slow and limited. However, by using suitable physicochemical means (good nutrient and O_2 supply, addition of electron acceptors, optimal temperature), the bioremediation process can be engineered for more efficient degradation of pollutants.

Advantages of in situ bioremediation:

1. Cost-effective, with minimal exposure to public or site personnel.
2. Sites of bioremediation remain minimally disrupted.

Disadvantages of in situ bioremediation:

1. Very time consuming process.
2. Sites are directly exposed to environmental factors (temperature, O_2 supply etc.).
3. Microbial degrading ability varies seasonally.

Ex Situ Bioremediation:

The waste or toxic materials can be collected from the polluted sites and the bioremediation with the requisite microorganisms (frequently a consortium of organisms) can be carried out at designed places. This process is certainly an improvement over in situ bioremediation, and has been successfully used at some places.

Advantages of ex situ bioremediation:

1. Better controlled and more efficient process.
2. Process can be improved by enrichment with desired microorganisms.
3. Time required in short.

Disadvantages of ex situ bioremediation:

1. Very costly process.
2. Sites of pollution are highly disturbed.
3. There may be disposal problem after the process is complete.

Metabolic Effects of Microorganisms on Xenobiotics:

Although it is the intention of the biotechnologist to degrade the xenobiotics by microorganisms to the advantage of environment and ecosystem, it is not always possible. This is evident from the different types of metabolic effects as shown below.

Detoxification:

This process involves the microbial conversion of toxic compound to a nontoxic one. Biodegradation involving detoxification is highly advantageous to the environment and population.

Activation:

Certain xenobiotics which are not toxic or less toxic may be converted to toxic or more toxic products. This is dangerous.

Degradation:

The complex compounds are degraded to simpler products which are generally harmless.

Conjugation:

The process of conjugation may involve the conversion of xenobiotics to more complex compounds. This is however, not very common.

Types of Reactions in Bioremediation:

Microbial degradation of organic compounds primarily involves aerobic, anaerobic and sequential degradation.

Aerobic bioremediation:

Aerobic biodegradation involves the utilization of O_2 for the oxidation of organic compounds. These compounds may serve as substrates for the supply of carbon and energy to the microorganisms. Two types of enzymes namely mono-oxygenases and- di-oxygenases are involved in aerobic biodegradation. Mono-oxygenases can act on both aliphatic and aromatic compounds while di-oxygenases oxidize aliphatic compounds.

Anaerobic bioremediation:

Anaerobic biodegradation does not require O_2 supply. The growth of anaerobic microorganisms (mostly found in solids and sediments), and consequently the degradation processes are slow. However, anaerobic biodegradation is cost- effective, since the need for continuous O_2 supply is not there. Some of the important anaerobic reactions and examples of organic compounds degraded are listed below.

Hydrogenation and dehydrogenation — benzoate, phenol, catechol.

Dehaiogenation — Polychlorinated biphenyls (PCBs), chlorinated ethylene's. The term de-chlorination is frequently used for dehaiogenation of chlorinated compounds.

Carboxylation and decarboxylation — toluene, cresol and benzoate.

Sequential Bioremediation:

In the degradation of several xenobiotics, both aerobic and anaerobic processes are involved. This is often an effective way of reducing the toxicity of a pollutant. For instance, tetra chloromethane and tetrachloroethane undergo sequential degradation.

Biodegradation of Hydrocarbons:

Hydrocarbon are mainly the pollutants from oil refineries and oil spills. These pollutants can be degraded by a consortium or cocktail of microorganisms e.g. Pseudomonas, Corynebacterium, Arthrobacter, Mycobacterium and Nocardia.

Biodegradation of Aliphatic Hydrocarbons:

The uptake of aliphatic hydrocarbons is a slow process due to their low solubility in aqueous medium. Both aerobic and anaerobic processes are operative for the degradation of aliphatic hydrocarbons. For instance, unsaturated hydrocarbons are degraded in both anaerobic and aerobic environments, while saturated ones are degraded by aerobic process. Some aliphatic hydrocarbons which are reclacitrant to aerobic process are effectively degraded in anaerobic environment e.g. chlorinated aliphatic compounds (carbon tetrachloride, methyl chloride, vinyl chloride).

Biodegradation of Aromatic Hydrocarbons:

Microbial degradation of aromatic hydrocarbons occurs through aerobic and anaerobic processes. The most important microorganism that participates in these processes is Pseudomonas.

The biodegradation of aromatic compounds basically involves the following sequence of reactions:

1. Removal of the side chains.
2. Opening of the benzene ring.

Most of the non-halogenated aromatic compounds undergo a series of reactions to produce catechol or protocatechuate. The bioremediation of toluene, L-mandelate, benzoate, benzene, phenol, anthracene, naphthalene, phenanthrene and salicylate to produce catechol is shown in

Fig. 59.1. Likewise, Fig. 59.2, depicts the bioremediation of quinate, p-hydroxymandelate, p-hydroxybenzoyl formate, p-toluate, benzoate and vanillate to produce protocatechuate.

Catechol and protocatechuate can undergo oxidative cleavage pathways. In ortho-cleavage pathway, catechol and protocatechuate form acetyl CoA (Fig. 59.3), while in meta-cleavage pathway (Fig. 59.4), they are converted to pyruvate and acetaldehyde. The degraded products of catechol and protocatechuate are readily metabolised by almost all the organisms.

Biodegradation of Pesticides and Herbicides:

Pesticides and herbicides are regularly used to contain various plant diseases and improve the crop yield. In fact, they are a part of the modern agriculture, and have significantly contributed to green revolution. The common herbicides and pesticides are propanil (anilide), propham (carbamate), atrazine (triazine), picloram (pyridine), dichlorodiphenyl trichloroethane (DDT) monochloroacetate (MCA), monochloropropionate (MCPA) and glyphosate (organophosphate). Most of the pesticides and herbicides are toxic and are recalcitrant (resistant to biodegradation). Some of them are surfactants (active on the surface) and retained on the surface of leaves.

Biodegradation of Halogenated Aromatic Compounds:

Most commonly used herbicides and pesticides are aromatic halogenated (predominantly chlorinated) compounds. The bio-degradative pathways of halogenated compounds are comparable with that described for the degradation of non-halogenated aromatic compounds (Figs. 59.1, 59.2, 59.3 and 59.4). The rate of degradation of halogenated compounds is inversely related to the number of halogen atoms that are originally present on the target molecule i.e. compounds with higher number of halogens are less readily degraded.

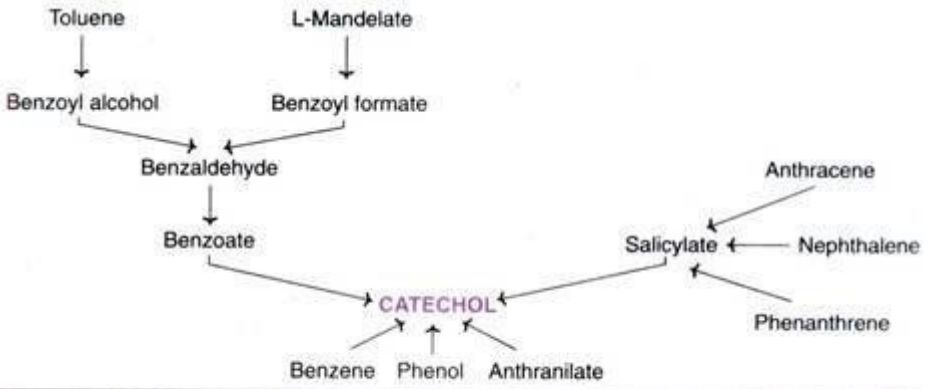


***Fig. 59.1** : Bioremediation of certain aromatic compounds by bacteria to produce catechol.*

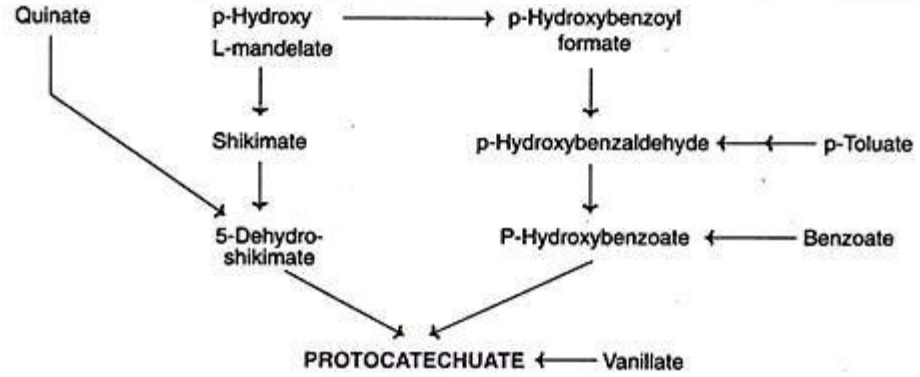


***Fig. 59.2** : Bioremediation of certain organic compounds by bacteria to produce protocatechuate.*

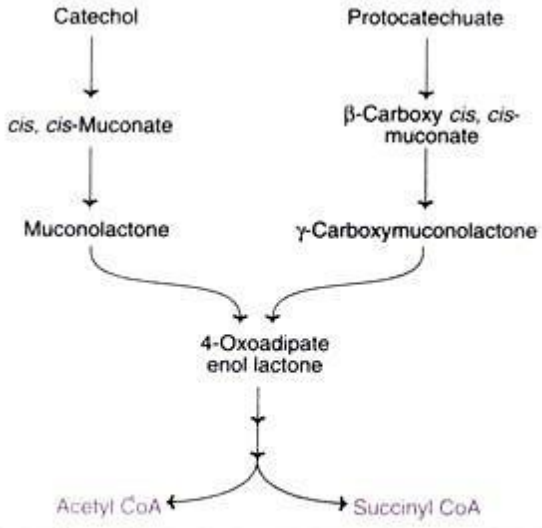


***Fig. 59.3** : Conversion of catechol and protocatechuate to acetyl CoA and succinate by **ortho–cleavage pathway**.*

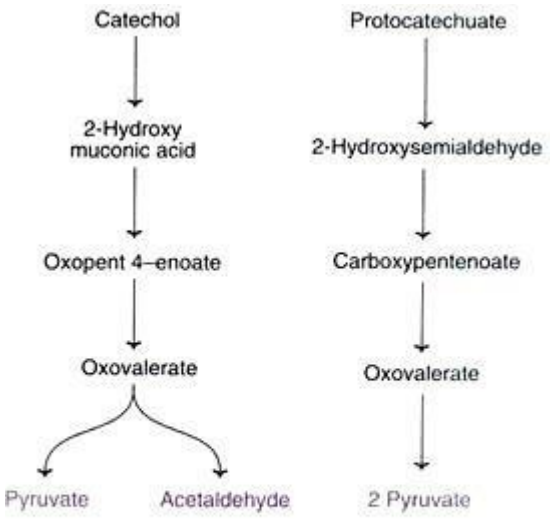


Fig. 59.4** : Conversion of catechol and protocatechuate to pyruvate and acetaldehyde by **meta–cleavage pathway.

Dehalogenation (i.e. removal of a halogen substituent from an organic compound) of halogenated compounds is an essential step for their detoxification. Dehalogenation is frequently catalysed by the enzyme di-oxygenase. In this reaction, there is a replacement of halogen on benzene with a hydroxyl group.

Most of the halogenated compounds are also converted to catechol and protocatechuate which can be metabolised (Fig. 59.4). Besides Pseudomonas, other microorganisms such as Azotobacter, Bacilluefs and E. coli are also involved in the microbial degradation of halogenated aromatic compounds.

Biodegradation of Polychlorinated Biphenyls (PCBs):

The aromatic chlorinated compounds possessing biphenyl ring (substituted with chlorine) are the PCBs e.g. pentachlorobiphenyl. PCBs are commercially synthesized, as they are useful for various purposes — as pesticides, in electrical conductivity (in transformers), in paints and adhesives. They are inert, very stable and resistant to corrosion.

However, PCBs have been implicated in cancer, damage to various organs and impaired reproductive function. Their commercial use has been restricted in recent years, and are now used mostly in electrical transformers.

PCBs accumulate in soil sediments due to hydrophobic nature and high bioaccumulation potential. Although they are resistant to biodegradation, some methods have been recently developed for anaerobic and aerobic oxidation by employing a consortium of

microorganisms. Pseudomonas, Alkali genes, Corynebacterium and Acinetobacter. For more efficient degradation of PCBs, the microorganisms are grown on biphenyls, so that the enzymes of biodegradation of PCBs are induced.

Biodegradation of Some Other Important Compounds:

Organo-nitro Compounds:

Some of the toxic organo-nitro compounds can be degraded by microorganisms for their detoxification.

2, 4, 6-Trinitrotoluene (TNT):

Certain bacterial and fungal species belonging to Pseudomonas and Clostrium can detoxify TNT.

Nitrocellulose:

Hydrolysis, followed by anaerobic nitrification by certain bacteria, degrades nitrocellulose.

Synthetic detergents:

They contain some surfactants (surface active agents) which are not readily biodegradable. Certain bacterial plasmid can degrade surfactants.

Genetic Engineering for More Efficient Bioremediation:

Although several microorganisms that can degrade a large number of xenobiotics have been identified, there are many limitations in bioremediation:

i. Microbial degradation of organic compounds is a very slow process.

ii. No single microorganism can degrade all the xenobiotics present in the environmental pollution.

iii. The growth of the microorganisms may be inhibited by the xenobiotics.

iv. Certain xenobiotics get adsorbed on to the particulate matter of soil and become unavailable for microbial degradation.

It is never possible to address all the above limitations and carry out an ideal process of bioremediation. Some attempts have been made in recent years to create genetically engineered microorganisms (CEMs) to enhance bioremediation, besides degrading xenobiotics which are highly resistant (recalcitrant) for breakdown. Some of these aspects are briefly described.

Genetic Manipulation by Transfer of Plasmids:

The majority of the genes responsible for the synthesis of bio-degradative enzymes are located on the plasmids. It is therefore logical to think of genetic manipulations of plasmids. New strains of bacteria can be created by transfer of plasmids (by conjugation) carrying genes for different degradative pathways.

If the two plasmids contain homologous regions of DNA, recombination occurs between them, resulting in the formation of a larger fused plasmid (with the combined functions of both plasmids). In case of plasmids which do not possess homologous regions of DNA, they can coexist in the bacterium (to which plasmid transfer was done).

The first successful development of a new strain of bacterium (Pseudomonas) by manipulations of plasmid transfer was done by Chakrabarty and his co-workers in 1970s. They used different plasmids and constructed a new bacterium called as superbug that can degrade a number of hydrocarbons of petroleum simultaneously.

United States granted patent to this superbug in 1981 (as per the directive of American Supreme Court). Thus, superbug became the first genetically engineered microorganism to be patented. Superbug has played a significant role in the development of biotechnology industry, although it has not been used for large scale degradation of oil spills.

Creation of Superbug by Transfer of Plasmids:

Superbug is a bacterial strain of Pseudomonas that can degrade camphor, octane, xylene and naphthalene. Its creation is depicted in Fig. 59.5.

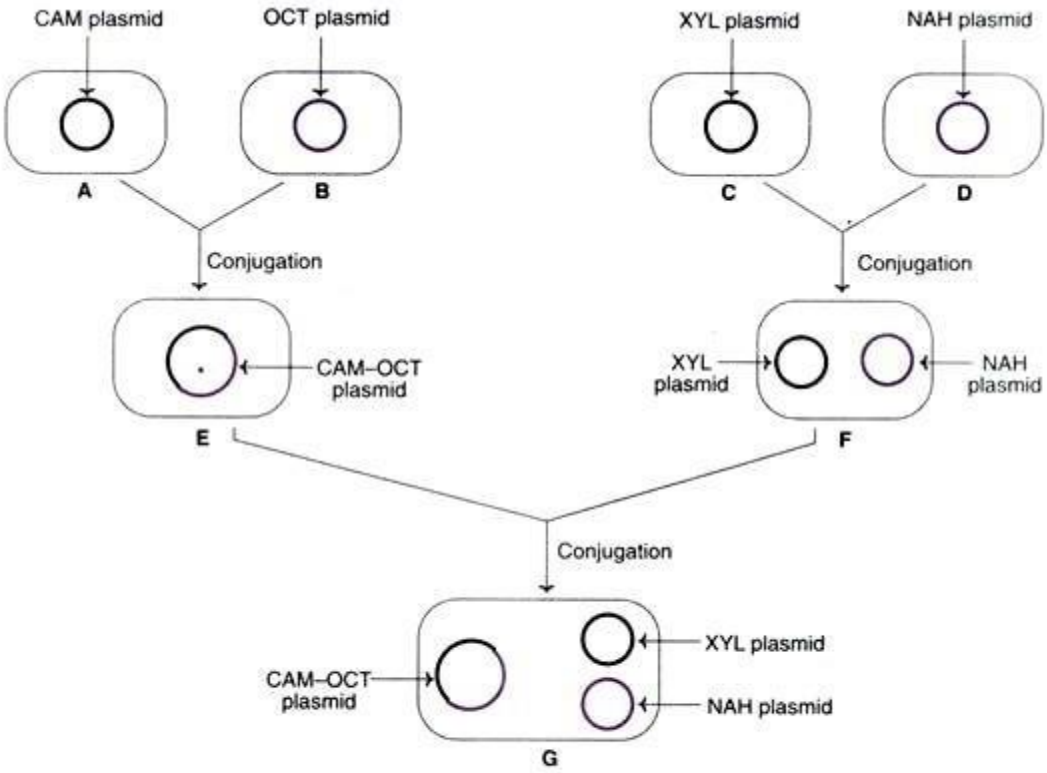


Fig. 59.5 : Creation of the superbug by transfer of plasmids (A, B, C, D, E, F and G are the different strains of bacteria containing the plasmids shown. Strain G is the superbug.)

The bacterium containing CAM (camphor- degrading) plasmid was conjugated with another bacterium with OCT (octane-degrading) plasmid. These plasmids are not compatible and therefore, cannot coexist in the same bacterium. However, due to the presence of homologous regions of DNA, recombination occurs between these two plasmids resulting in a single CAM-OCT plasmid. This new bacterium possesses the degradative genes for both camphor and octane.

Another bacterium with XYL (xylene-degrading) plasmid is conjugated with NAH (naphthalene- degrading) plasmid containing bacterium. XYL and NAH plasmids are compatible and therefore can coexist in the same bacterium. This newly, produced bacterium contains genes for the degradation of xylene and naphthalene.

The next and final step is the conjugation of bacterium containing CAM-OCT plasmid with the other bacterium containing XYL and NAH plasmids. The newly created strain is the superbug that carries CAM-OCT plasmid (to degrade camphor and octane), XYL (xylene-degrading) plasmid and NAH (naphthalene-degrading) plasmid.

Development of Salicylate—Toluene Degrading Bacteria by Plasmid Transfer:

Some attempts have been made for the creation of a new strain of the bacterium Pseudomonas putida to simultaneously degrade toluene and salicylate. Toluene-degrading (TOL) plasmid was transferred by conjugation to another bacterium that is capable of degrading salicylate (due to the presence of SAL plasmid).

The newly developed strain of Pseudomonas can simultaneously degrade both toluene and salicylate. And this occurs even at a low temperature (0-5°C). However, the new bacterium is not in regular use, as more research is being conducted on its merits and demerits.

Genetic Manipulation by Gene Alteration:

Work is in progress to manipulate the genes for more efficient biodegradation. The plasmid pWWO of Pseudomonas codes for 12 different enzymes responsible for the meta-cleavage pathway (for the conversion of catechol and protocatechuate to pyruvate and acetaldehyde, for degradation of certain aromatic compounds. Some success has been reported to alter the genes of plasmid pWWO for more efficient degradation of toluene and xylene.

Genetically Engineered Microorganisms (GEMs) in Bioremediation:

Superbug is the first genetically engineered microorganism. Several workers world over have been working for the creation of GEMs, specifically designed for the detoxification of xenobiotics. A selected list of GEMs with a potential for the degradation of xenobiotics is given in Table 59.3. Almost all these CFMs have been created by transferring plasmids.

TABLE 59.3 A selected list of genetically engineered microorganisms (GEMs) with the potential xenobiotics that can be degraded

Genetically engineered microorganism (GEMs)	*Xenobiotic*
Pseudomonas diminuta	Parathion
P. oleovorans	Alkane
P. cepacia	2, 4, 5-Trichlorophenol
P. putida	Mono- and dichloro-aromatic compounds
Alcaligenes sp	2, 4-Dichlorophenoxy acetic acid
Acinetobacter sp	4-Chlorobenzene

Bio-surfactant Producing GEM:

A genetically engineered Pseudomonas aeruginosa has been created (by Chakarabarty and his group). This new strain can produce a glycolipid emulsifier (a bio-surfactant) which can

reduce the surface tension of an oil water interface. The reduced interfacial tension promotes biodegradation of oils.

GEM for Degradation of Vanillate and SDS:

A new strain of Pseudomonas sp (strain ATCC 1915) has been developed for the degradation of vanillate (waste product from paper industry) and sodium dodecyl sulfate (SDS, a compound used in detergents).

GEMs and Environmental Safety:

The genetically engineered microorganisms (GEMs) have now become handy tools of biotechnologists. The risks and health hazards associated with the use of GEMs are highly controversial and debatable issues. The fear of the biotechnologists and even the general public is that the new organism (GEM), once it enters the environment, may disturb the ecological balance and cause harm to the habitat. Some of the GEMs may turn virulant and become genetic bombs, causing great harm to humankind.

Because of the risks involved in the use of GEMs, so far no GEM has been allowed to enter the environmental fields. Thus, the use of GEMs has been confined to the laboratories, and fully controlled processes of biodegradation (usually employing bioreactors). Further, several precautionary measures are taken while creating GEMs, so that the risks associated with their use are minimal.

Some researchers are of the opinion that GEMs will create biotechnological wonders for the environmental management of xenobiotics, in the next few decades. This may be possible only if the associated risks of each GEM is thoroughly evaluated, and fully assured of its biosafety.

Bioremediation of Contaminated Soils and Waste Lands:

Due to industrialization and extensive use of insecticides, herbicides and pesticides, the solids and waste lands world over are getting polluted. The most common pollutants are hydrocarbons, chlorinated solvents, polychlorobiphenyls and metals.

Bioremediation of soils and waste lands by the use of microorganisms is gaining importance in the recent years. In fact, some success has been reported for the detoxification of certain pollutants (e.g. hydrocarbons) in the soil by microorganisms. Bioremediation of soils can be done by involving two principles-bio-stimulation and bio-augmentation.

Bio-stimulation in Soil Bioremediation:

Bio-stimulation basically involves the stimulation of microorganisms already present in the soil, by various means.

This can be done by many ways:

i. Addition of nutrients such as nitrogen and phosphorus.

ii. Supplementation with co-substrates e.g. methane added to degrade trichloroethylene.

iii. Addition of surfactants to disperse the hydrophobic compounds in water.

Addition of nutrient and co-substrates promote microbial growth while surfactants expose the hydrophobic molecules. In all these situations, the result is that there occurs bio-stimulation by effective bioremediation of polluted soil or waste land.

Bio-augmentation in Soil Bioremediation:

Addition of specific microorganisms to the polluted soil constitutes bio-augmentation. The pollutants are very complex molecules and the native soil microorganisms alone may not be capable of degrading them effectively. The examples of such pollutants include polychlorobiphenyls (PCBs), trinitrotoluene (TNT), polyaromatic hydrocarbons (PAHs) and certain pesticides.

Based on the research findings at the laboratory level (with regard to biodegradation), it is now possible to add a combination of microorganisms referred to as consortium or cocktail of microorganisms, to achieve bio-augmentation.

With the development of genetically engineered microorganisms (GEMs), they can be also used to bio-augment soils for very efficient bioremediation. But the direct use of GEMs in the soils is associated with several risks and health hazards.

Techniques of Soil Bioremediation:

The most commonly used methods for the bioremediation are soils are in situ bioremediation, land farming and slurry phase bioreactors.

In Situ Bioremediation of Soils:

In situ bioremediation broadly involves the biological clean-up of soils without excavation. This technique is used for the bioremediation of sub-surfaces of soils, buildings and road ways that are polluted. Sometimes, water (oxygenated) is cycled through the sub-surfaces for increasing the efficiency of microbial degradation. There are two types of in situ soil bioremediation techniques- bioventing and phytoremediation.

Bioventing:

This is very efficient and cost- effective technique for the bioremediation of petroleum contaminated soils. Bioventing involves aerobic biodegradation of pollutants by circulating air through sub-surfaces of soil. Although, it takes some years, bioventing can be used for the degradation of soluble paraffin's and polyaromatic hydrocarbons. The major limitation of this technique is air circulation which is not always practicable.

Phytoremediation:

Bioremediation by use of plants constitutes phytoremediation. Specific plants are cultivated at the sites of polluted soil. These plants are capable of stimulating the biodegradation of pollutants in the soil adjacent to roots (rhizosphere) although phytoremediation is a cheap and environmental friendly clean-up process for the biodegradation of soil pollutants, it takes several years.

Land farming in Soil Bioremediation:

Land farming is a technique for the bioremediation of hydrocarbon contaminated soils. A diagrammatic representation of land farming system (also referred to as solid phase soil reactor) is depicted in Fig. 59.6.

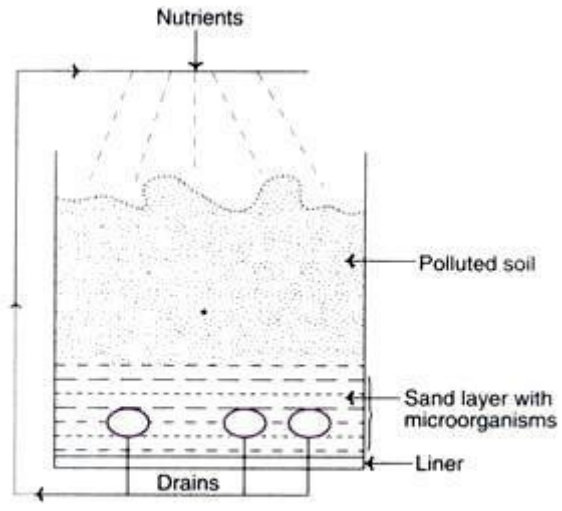


Fig. 59.6 : A diagrammatic representation of landfarming system.

The soil is excavated, mixed with microorganisms and nutrients and spread out on a liner, just below the polluted soil. The soil has to be regularly ploughed for good mixing and aeration. If the soil is mixed with compost and/or temperature is increased the efficiency of biodegradation increases.

Addition of co-substrates, and anaerobic pretreatment of polluted soils also increases the degradation process. Land farming has been successfully used for the bioremediation of soils polluted with chloroethane benzene, toluene and xylene. The last three compounds are often referred to as BTX aromatics.

Slurry-phase Bioreactors in Soil Bioremediation:

Slurry-phase bioreactors are improved land farming systems. In these cases, the excavated polluted soil is subjected to bioremediation under optimally controlled conditions in specifically designed bioreactors. Due to a close contact between the xenobiotics and the microorganisms, and the optimal conditions (nutrient supply, temperature, aeration etc.), the degradation is very rapid and efficient. Slurry-phase bioreactors, however, are not suitable for widespread use due to high cost.

Bioremediation of Ground Water:

Environmental pollution also results in the contamination of ground water at several places. The commonly found pollutants are the petroleum hydrocarbons (aliphatic, aromatic, cyclic and substituted molecules). Bioremediation of ground water can be carried out by two methods pump- and -treat technique and bio-fencing technique.

Pump and Treat Technique for Bioremediation of Ground Water:

Bioremediation of underground water by pump- and -treat technology is mostly based on physicochemical principles to remove the pollutants. The treatment units are set up above the ground. Strip columns and activated carbon filters can remove most of the ground water pollutants. Treated water is recycled through injection well several times so that the pollutants are effectively removed.

For removal of certain organic pollutants, biological reactors (bioreactors) have to be installed (Fig. 59.7A). For instance, for the biodegradation of tetrachloroethane, a bioreactor with granular methogenic sludge is found to be effective. In recent years, bioreactors with both aerobic and anaerobic bacteria have been developed for better bioremediation of highly polluted ground waters.

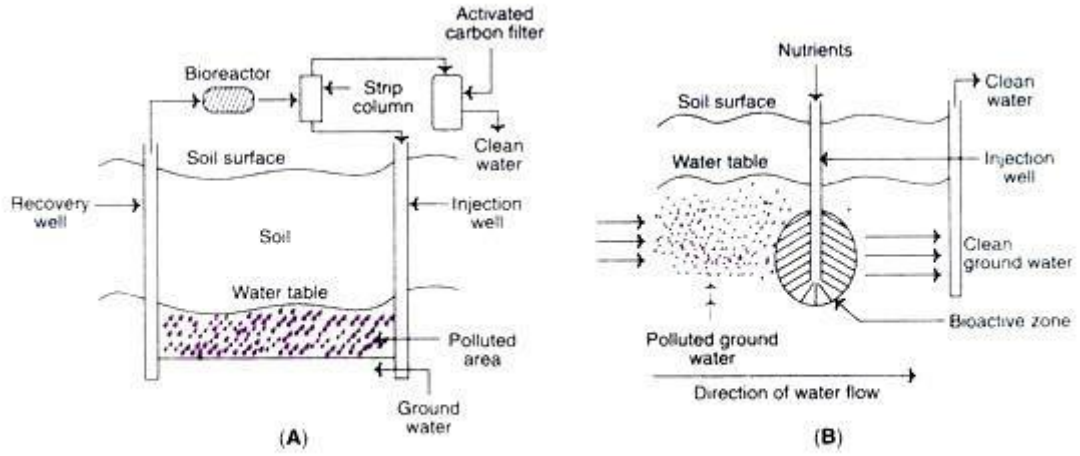


Fig. 59.7 : Bioremediation of ground water (A) Pump- and -treat technique (B) Biofencing technique.

It is however, not possible to achieve good clean up of ground water by pump- and -treat technology, for various reasons (sub-surface heterogeneities, strongly adsorbed compounds, low permeability of pollutants etc.).

Bio-fencing Technique for Bioremediation of Ground Water:

Bio-fencing is an improved technique for the bioremediation of ground water. It consists of installation of a bioactive zone at the down- gradient edge of a contaminated ground water area. Nutrients are injected through a well to the bioactive zone (Fig. 59.7B). As the ground water passes through the bioactive zone (by the impact of natural direction of flow), the pollutants are biodegraded, and clean ground water comes out.

UNIT IV

Production of industrial important enzymes

Industrial enzymes are enzymes that are commercially used in a variety of industries such as pharmaceuticals, chemical production, biofuels, food & beverage, and consumer products. Due to advancements in recent years, biocatalysis through isolated enzymes is considered more economical than use of whole cells. Enzymes may be used as a unit operation within a process to generate a desired product, or may be the product of interest. Industrial biological catalysis through enzymes has experienced rapid growth in recent years due to their ability to operate at mild conditions, and exceptional chiral and positional specificity, things that traditional chemical processes lack.[1] Isolated enzymes are typically used in hydrolytic and isomerization reactions. Whole cells are typically used when a reaction requires a co-factor. Although co-factors may be generated in vitro, it is typically more cost-effective to use metabolically active cells.[1]

- 

Enzymes as a Unit Operation		
Enzyme	**Industry**	**Application**
Palatase	Food	Enhance cheese flavor
Lipozyme TL IM	Food	Interesterification of vegetable oil
Lipase AK Amano	Pharmaceutical	Synthesis of chiral compounds
Lipopan F	Food	Emulsifier
Cellulase	Biofuel	Class of enzymes that degrade cellulose to glucose monomers
Amylase	Food/biofuel	Class of enzymes that degrade starch to glucose monomers
Xylose isomerase	Food	High fructose corn syrup production
Resinase	Paper	Pitch control in paper processing

Penicillin amidase[11]	Pharmaceutical	Synthetic antibiotic production
Amidase	Chemical	Class of enzymes used for non-proteinogenic enantiomerically pure amino acid production

Enzymes as a desired product

To industrialize an enzyme, the following upstream and downstream enzyme production processes are considered:

Upstream

Upstream processes are those that contribute to the generation of the enzyme.

Selection of a suitable enzyme

An enzyme must be selected based upon the desired reaction. The selected enzyme defines the required operational properties, such as pH, temperature, activity, and substrate affinity.

Identification and selection of a suitable source for the selected enzyme

The choice of a source of enzymes is an important step in the production of enzymes. It is common to examine the role of enzymes in nature and how they relate to the desired industrial process. Enzymes are most commonly sourced through bacteria, fungi, and yeast. Once the source of the enzyme is selected, genetic modifications may be performed to increase the expression of the gene responsible for producing the enzyme.

Process development

Process development is typically performed after genetic modification of the source organism, and involves the modification of the culture medium and growth conditions. In many cases, process development aims to reduce mRNA hydrolysis and proteolysis.

Large scale production[edit]

Scaling up of enzyme production requires optimization of the fermentation process. Most enzymes are produced under aerobic conditions, and as a result, require constant oxygen input, impacting fermenter design. Due to variations in the distribution of dissolved oxygen, as well as temperature, pH, and nutrients, the transport phenomena associated with these parameters must be considered. The highest possible productivity of the fermenter is achieved at maximum transport capacity of the fermenter.

Downstream

Downstream processes are those that contribute to separation or purification of enzymes.

Removal of insoluble materials and recovery of enzymes from the source

The procedures for enzyme recovery depend on the source organism, and whether enzymes are intracellular or extracellular. Typically, intracellular enzymes require cell lysis and separation of complex biochemical mixtures. Extracellular enzymes are released into the culture medium, and are much simpler to separate. Enzymes must maintain their native conformation to ensure their catalytic capability. Since enzymes are very sensitive to pH, temperature, and ionic strength of the medium, mild isolation conditions must be used.[12]

Concentration and primary purification of enzymes

Depending on the intended use of the enzyme, different levels purity are required. For example, enzymes used for diagnostic purposes must be separated to a higher purity than bulk industrial enzymes to prevent catalytic activity that provides erroneous results. Enzymes used for therapeutic purposes typically require the most rigorous separation. Most commonly, a combination of chromatography steps is employed for separation.

The purified enzymes are either sold in pure form and sold to other industries, or added to consumer goods.

Enzymes as a Desired Product		
Enzyme	**Industry**	**Application**
Novozym-435	Consumer Goods	Isopropyl myristate production (Cosmetic)
Bromelain	Food	Meat tenderizer
Noopazyme	Food	Improve noodle quality
Asparaginase	Pharmaceutical	Lymphatic cancer therapeutic
Ficin	Pharmaceutical	Digestive aid
Urokinase	Pharmaceutical	Anticoagulant
β-Lactamase	Pharmaceutical	Penicillin allergy treatment
Subtilisin	Consumer Goods	Laundry detergent

BIOFERTILIZERS

Biofertilizers are the substance that contains microorganism's living or latent cells. Biofertilizers increase the nutrients of host plants when applied to their seeds, plant surface or soil by colonizing the rhizosphere of the plant. Biofertilizers are more cost-effective as compared to chemical fertilizers.

- Application of Microbes

Over the years, chemical fertilizers have helped farmers increase crop production to meet the increasing demand. However, the overuse of these fertilizers is harmful because they cause air and water pollution; and also deplete minerals from the soil. Therefore, there is a need to now switch to organic farming which involves the use of 'Biofertilizers'. Let's learn more about biofertilizers and some of their types.

Biofertilizers

'Bio' means 'life'. Therefore, by definition biofertilizers are living organisms that enrich the nutrient quality of the soil. It refers to the use of microbes instead of chemicals to enhance the nutrition of the soil. As a result, it is also less harmful and does not cause pollution.

This is what makes them so important to organic farming because they are completely environment-friendly. Let's take a look at the different microbes that are used as biofertilizers.

Types of Biofertilizers:

1. Bacteria
2. Fungi
3. Cyanobacteria

Bacteria

The nitrogen-fixing nodules on the roots of legumes is a great example of biofertilizers. The nodules are formed by the association of the bacterium '*Rhizobium'* with the roots of these plants. This association is beneficial and is, therefore, called 'symbiotic'.

The nodules help in fixing atmospheric nitrogen into organic forms which can then be used as nutrition by the plants. Adding *Rhizobium* cultures to fields has become a common practice to ensure an adequate amount of nitrogen in the soil.

Other examples of bacteria that act as biofertilizers include Azospirillum and Azotobacter. These bacteria are free-living in the soil. Azotobacter is usually used with crops like cotton, wheat, mustard, maize, etc.

Fungi

Symbiotic associations exist between plants and fungi too. These associations are called 'Mycorrhizae'. The fungus in this association absorbs phosphorus from the soil and provides it to the plant. Plants that grow with these associations also show other advantageous characteristics such as:

1. Tolerance to drought conditions and salinity.
2. Resistance to root-borne pathogens.
3. An overall increase in plant growth and development.

Cyanobacteria

These are blue-green bacteria found in water and on land. They also help fix atmospheric nitrogen. Examples are *Oscillatoria, Nostoc, Anabaena* etc. The symbiotic association between the aquatic fern Azolla and *Anabaena* is very important for rice fields. In this association, *Anabaena* receives carbon and nitrogen from the plant in exchange for fixed nitrogen. This adds organic matter to the soil enhancing the fertility of rice fields.

Nowadays, many biofertilizers are commercially available in the market for farmers to buy and use. These not only help replenish the soil nutrients but also reduce the dependency on chemical fertilizers. This helps in maintaining the mineral content of the soil and reduces pollution to a great extent.

Introduction of Biopesticides

Biopesticides are types of chemicals extracted from natural materials such as plants, animals, bacteria or certain minerals and these chemicals can be used for controlling pests. For example, canola oil/baking soda with pesticidal applications are considered biopesticides. As costs of using synthetic chemicals became apparent, there was resurgence in academic and industrial research of biopesticide. Development of new and useful biopesticides has continued to increase rapidly since the mid-1990s. In fact, more than 100 biopesticide active ingredients have been registered in the U.S. since 1995. Many of these have been introduced commercially in a variety of products1 .

Biopesticides can be considered as dividing into three major classes2 :

1. Microbial pesticides consist of microorganism (e.g. bacterium, fungus, virus or protozoan) as the active ingredient. Microbial pesticides can control many different kinds of pests, although each separate active ingredient is relatively specific for its target pest(s). For example, there are fungi that can control certain weeds, and other fungi that can kill specific insects.

2. Biochemical pesticides are naturally occurring substances that control pests by non-toxic mechanisms. Conventional pesticides, by contrast, are generally synthetic materials that directly kill or inactivate the pest. Biochemical pesticides include substances, such as insect sex pheromones, which interfere with mating, as well as various scented plant extracts that attract insect pests to traps. Because it is sometimes difficult to determine whether a substance meets the criteria for classification as a biochemical pesticide, responsible authority would establish a special committee to make such decisions.

3. Plant-Incorporated-Protectants (PIPs) are pesticidal substances that plants produce from genetic material that has been added to the plant. For example, scientists can take the gene for the B.t. pesticidal protein, and introduce the gene into the plant's own genetic material. Then the plant, instead of the B.t. bacterium, manufactures the substance that destroys the pest. Biopesticides products, compared to broad spectrum chemical pesticides, are usually more target specific and inherently less toxic which would cause less impact to other non-target species, such as other insects, birds and mammals, while application. In addition, biopesticides are often effective in low application rate and could decompose quickly in natural environment. This leads to lower exposures and largely avoid the pollution problems

caused by conventional pesticides. Besides, chance of pests developing resistance is much lowered.

Single cell protein production

Single cell protein (SCP) is not pure protein (Table 1), but refers to the whole cells of bacteria, yeasts, filamentous fungi or algae, and also contains carbohydrates, lipids, nucleic acids, mineral salts and vitamins. It has several advantages over conventional plant and animal protein sources, which include:

1. rapid growth rate and high productivity;
2. high protein content, 30–80% on a dry weight basis;
3. the ability to utilize a wide range of low cost carbon sources, including waste materials;
4 .strain selection and further development , as these organisms are amenable to genetic modification;
5. the processes take little land area;
6. production is independent of seasonal and climatic variations;
7. consistent product quality.

Table 1: Protein and nucleic acid content in microorganisms

Microbe	Protein percentage	Nucleic acid
Bacteria	50–85	10–16%
Yeast	45–55	5–12%
Filamentous fungi	30–55	3–10%
Algae	45–65	4–6%

Note: soya beans contain approximately 40% protein.

The protein content and quality is largely dependent on the specific microorganism utilized and the fermentation process .

Fast-growing aerobic microorganisms are primarily used due to their high yields and high productivity. Bacteria generally have faster growth rates and can grow at higher temperatures than yeasts or filamentous fungi, and normally contain more protein. Yeasts grow relatively rapidly and, like bacteria, their unicellular character gives somewhat fewer fermentation problems than do filamentous organisms. However, many filamentous fungi have a capacity to degrade a wide range of materials and, like yeasts, can tolerate a low pH, which reduces the risk of microbial contamination. They are also more easily harvested at the end of

fermentation than yeasts or bacteria. Selection of a suitable microbial strain for SCP production must take several characteristics into account, including:

1. Growth rate, productivity and yields on the specific, low-cost, substrates to be used;
2. Temperature and pH tolerance;
3. Oxygen requirements, heat generation during fermentation and foaming characteristics;
4. Growth morphology and genetic stability in the fermentation;
5. Ease of recovery of SCP and requirements for further downstream processing;
6 .structure and composition of the final product, in terms of protein content, amino acid profile, RNA level, flavor, aroma, color and texture.

Other major factors are safety and acceptability. Most SCP products are used as animal feed and not for human consumption. These products must meet safety requirements. Obtaining regulatory approval for the production of proteins for human consumption is an even lengthier and more expensive process, and obviously influences the choice of production organism.

A safety aspect that must be considered for all SCP products is nucleic acid content. Many microorganisms have naturally high levels, because fermentation conditions favoring rapid growth rates and high protein content also promote elevated RNA levels. This can be problematic as the digestion of nucleic acids by humans and animals leads to the generation of purine compounds. Their further metabolism results in elevated plasma levels of uric acid, which may crystallize in the joints to give gout-like symptoms or forms kidney stones. Slow digestion or indigestion of some microbial cells within the gut and any sensitivity or allergic reactions to the microbial protein must also be examined. An additional concern is the absorption of toxic or carcinogenic substances, such as polycyclic aromatic compounds, which may be derived from certain growth substrates.

Single cell protein production processes The SCP production processes essentially contain the same basic stages of the carbon substrate or microorganism used.

1. Medium preparation. The main carbon source may require physical or chemical pretreatment prior to use.
2. Fermentation. Continuous fermentations are generally used, which are operated at close to the organism’s maximum growth, to fully exploit the superior productivity of continuous culture.

3. Separation and downstream processing. The cells are separated from the spent medium by filtration or centrifugation and may be processed in order to reduce the level of nucleic acids. RNase activity is retained and degrades RNA to nucleotides that diffuse out of the cells. Depending upon the growth medium used, further purification may be required, such as a solvent wash, prior to pasteurization, dehydration and packaging.

The physiological problems that are often encountered on scale-up include difficulties with:

1. Oxygen requirements and oxygen transfer rates;
2. Nutrient and temperature gradients;
3 .effects of CO2, as high levels may inhibit respiration in certain microorganisms;
4. Hydraulic pressure in deep fermenters.

MICROORGANISMS USED IN SCP PRODUCTION

Organisms to be used in SCP production should have the following properties:

(a) Absence of pathogenicity and toxicity
(b) (b) Protein quality and content: The amount of protein in the organisms should not only be high but should contain as much as possible of the amino acids required by man.
(c) (c) Digestibility and organoleptic qualities
(d) (d) Growth rate: It must grow rapidly in a cheap, easily available medium.
(e) (e) Adaptability to unusual environmental conditions

Bacteria

Methanomones sp.
Methylococius capsulatus
Pseudononas sp.
Hyphomisobium sp. Mixed
Acinetobacter sp.
Flavobacterium sp.
Arthrobacter simplex
Nocardia paraffinica
Nocardia paraffinica

Mycobacterium phlei
Nocardia sp.

Fungi

(f) Candida tropicals , Candida lipolytica ,Fusarium sp. and Aspergillus sp.

Mushrooms:

Mushrooms are the fruit bodies of edible fungi, commonly belonging to Basidiomycotina (Agaricus campestris, A. brunnescens, Pleurotus sajor-caju, Volvariella volvacea etc.) and rarely to Ascomycotina (Morchella conica, M. esculenta).

The mushrooms were used as food since long back, probably from 3000 B.C. as per ancient Indian literature. Since that time, the mushrooms are being consumed in different countries like Greece, Egypt, France etc. The Greeks and Romans described mushrooms as "food for the god". During that period, people consumed the mushrooms after collecting them from their natural habitat.

The cultivation was started in the early part of 18th century in France, but it became a thriving industry only by 1850 in Paris. In India, the first successful experimental cultivation of mushroom (A. bisporus) was initiated at Solan (Himachal Pradesh) in 1961. Later on, the cultivation of mushrooms gradually becomes popular with edible mushrooms in different parts of our country.

Assessment of nutritional value of mushrooms has been demonstrated that they complement and supplement the human diet and these are considered as "delight of the diabetic". Mushrooms have rightly been referred to as the "ultimate health food".

But there is no definite test to confirm whether a mushroom is poisonous or edible. Alexopoulos, Mims and Blackwell (1996) expressed the warning in their book "Introductory Mycology" to the Mushroom-consumers that "you can commit many mistakes in your life, but of consuming a poisonous mushroom only once".

Value of Mushrooms:

1. Nutritive Value:

Mushrooms became popular for their food value. The food values of mushrooms are as follows:

i. Mushrooms are the richest source of vegetable protein.

ii. The protein content varies from 1.1-4.98% in common cultivable mushroom (much higher than pulses, vegetables and fruits).

iii. All the essential amino acids including lysine (550 mg/gm) are present in much higher amount than even egg.

iv. Mushrooms contain sufficient quantities of mineral elements such as Na, K, Ca, P and Fe.

v. Mushrooms contain folic acid.

vi. Mushrooms contain vitamins like B, C, D and K.

vii. They contain little amount of fat (0.35- 0.65% dry wt.) and starch (0.02% dry wt.).

Different chemical contents of mushrooms and other foods are given in Table 4.15-4.18:

Table 4.15 : Protein content and energy value of mushrooms and other vegetables

	Name of the material	Protein content (% on dry weight basis)	Energy value (Kcal)
1.	Mushrooms	26.9	16
2.	Green peas	26.1	98
3.	Green beans	21.6	35
4.	Cabbage	18.4	24
5.	Cauliflower	28.8	25
6.	Beet root	12.9	42
7.	Potato	7.6	83
8.	Brinjal	15.1	24

Table 4.16 : Protein content of some cultivated mushrooms

	Name of mushroom	Protein content (% on dry weight basis)
1.	*Agaricus brunnescens*	3.94
2.	*Pleurotus ostreatus*	2.74
3.	*P. florida*	3.7
4.	*P. sajor-caju*	3.7
5.	*Volvariella volvacea*	4.98
6.	*Lentinus edodes*	1.7
7.	*Auricularia auricular*	1.1
8.	*Fammulina velutipes*	2.2

Table 4.17 : Chemical composition and energy value of some common mushrooms (% on fresh weight basis)

	Name of Mushroom	Water	Ash	Protein	Fat	Crude fibre	Energy Value (cal)
1.	*Agaricus brunnescens*	89.5	1.25	3.94	0.19	1.09	34.4
2.	*Pleurotus ostreatus*	92.5	0.97	2.47	0.65	1.08	23.1
3.	*Volvariella volvacea*	88.4	1.46	4.98	0.74	1.38	29.5
4.	*Termitomyces*	91.3	0.81	4.1	0.22	1.13	–

Table 4.18 : Composition of vitamins and minerals of some edible mushrooms (mg/100 gm dry weight)

	Name of Mushroom	Thiamin	Niacin	Riboflavin	Ascorbic acid	Ca	K	Na	Fe	P
1.	*Agaricus brunnescens*	1.1	55.7	5.0	81.9	23	4762	–	0.2	1429
2.	*Pleurotus ostreatus*	4.8	108.7	4.7	00.0	98	–	61	8.5	479
3.	*Volvariella volvacea*	1.2	91.9	3.3	20.2	71	3455	374	15.2	677

2. Medicinal Value:

Most of the mushrooms have high medicinal value to reduce blood pressure, obesity (to be fatty), constipation, atherosclerosis (fat deposition inside blood vessel) etc. Medicinal value of some mushrooms are given in Table 4.19.

Table 4.19 : Role of some edible fungi in controlling human diseases

	Name of Mushroom	Role in controlling diseases
1.	*Agaricus brunnescens* (syn. *A. bisporus*)	Stimulating digestion, Curing hypertension, Hyperacidity, Obesity, Constipation and Atherosclerosis (fat deposition inside the blood vessel).
2.	*Auricularia polytricha* (wood ear mushroom)	Strengthening health, helping blood circulation, using for the treatment of stomach ailments (sufferings), Piles, Atherosclerosis.
3.	*Pleurotus ostreatus*	Relaxing muscle, Joint pains, Antitumorous activity.
4.	*Pleurotus sajor-caju*	Reducing the rate of nephron detoriation (renal failure).
5.	*Volvariella volvacea*	Reducing blood pressure.

3. Biological Value:

Biologically mushrooms are very much important. The biological value includes nutritive value, medicinal value and their efficiency in degradation of substrate.

Cultivation Procedure of Mushrooms:

It is the technique to develop the fruit bodies of edible fungi. About a dozen fungi are cultivated in 100 countries with an annual production of 2.2 million tonnes. The common four genera are Agaricus, Lentinus, Volvariella and Pleurotus.

Together, they yield a major share of the total production. Out of many, Agaricus brunnescens (syn. A. bisporus), white button yields 56%; Lentinus edodes, shiitake yields 14%; Volvariella volvacea, paddy straw yields 8% and Pleurotus spp., oyster 7.7%.

The cultivation procedure of some of the mushrooms is given:

A. Cultivation of Agaricus Brunnescens (Syn. A. Bisporus):

The Agaricus brunnescens (syn. A. bisporus) is commonly known as white button mushroom (Fig. 4.107, 4.108). It contributes a major share in the mushroom production of the world. It is a temperate mushroom and can grow well in temperate conditions. Optimum temperature, optimum moisture, proper ventilation and good quality of spawn are very essential prerequisites for mushroom growth.

These are:

a. The optimum temperature for the mycelial growth is 24°C, while it is 14-18°C for the formation and development of fruit body.

b. Optimum moisture requires nearly at the saturation point. However, direct application of excess water in bed is harmful for the growing crop.

c. Proper ventilation is essential to remove toxic gases by the introduction of adequate fresh air.

d. Good quality of spawn i.e., the spawn should be prepared from the tissue of single fruit body and its productive capacity should be good enough.

The cultivation procedure is:

1. Production of spawn,
2. Preparation of compost,
3. Filling of trays with compost,
4. Spawning i.e., inoculation of compost,
5. Watering of inoculated compost filled trays,
6. Casing,
7. Harvesting of mushrooms (fruit bodies), and
8. Storage of mushrooms.

1. Production of Spawn:

The spawn (seed of mushroom) is a pure culture of the mycelia grown on a special medium. The medium is prepared by the grains of wheat, rye, sorghum or bajra along with some ingredients.

The preparation of spawn mainly consists of three steps:

a. Preparation of substrate,

b. Inoculation of substrate, and

c. Incubation of inoculated substrate for spawn production.

Preparation of Substrate:

Take 900 gms of grains (wheat or sorghum) in 600-900 ml of water in a container and boil for 15-20 minutes, After boiling, decant the excess water and allow the grains to surface drying by spreading on polythene sheet in shade for a few hours.

The grains are then mixed with chemicals like 2% calcium sulphate (gypsum) and 0.5% calcium carbonate (chalk) on dry weight basis and adjust the pH of the grain at 7-7.8. About 300-350 gms grains were then filled in milk bottles/ polypropylene bags.

Place a ring of tin (3.5 cm height and 3 cm diametre) towards the inner side of the open-end of polypropylene bag, tighten it with rubber band and then push the margin of the bag

towards the inner side and thus a mouth is prepared.

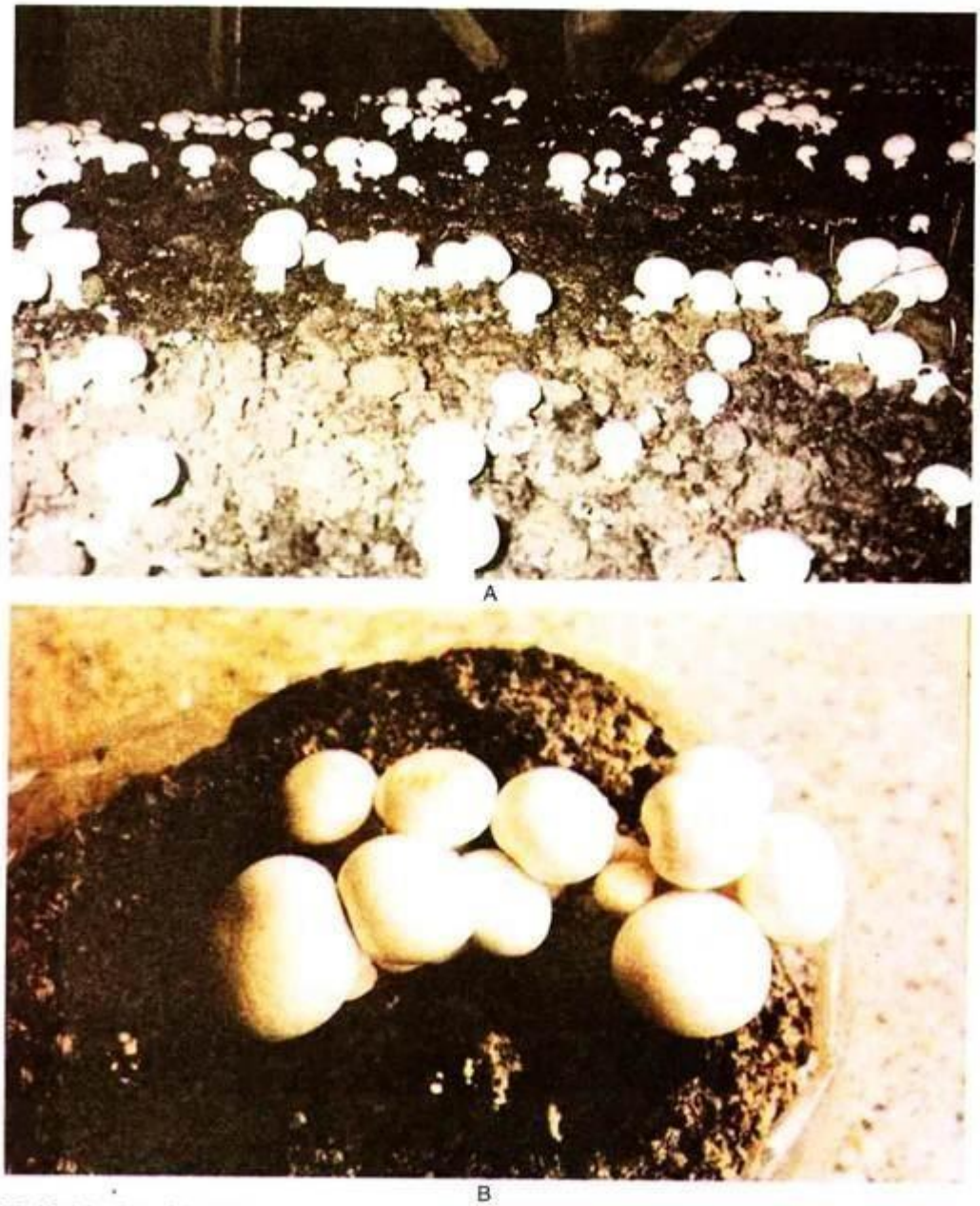


Fig. 4.107 : Fruit bodies of *Agaricus brunnescens* (syn. *A. bisporus*) on mushroom bed : A. Young fruit bodies (30 days after spawning) during mass production, B. Young fruit bodies (38 days after spawning)

[Courtesy : Dr. Samir Datta]

Plug the mouth of the bottle and/or polypropylene bag with non-absorbent cotton. Then cover the mouth with brown paper and tighten it with rubber band. Sterilise the substrate by autoclave at 15lb pressure for 30 minutes for 2 consecutive days. Kept the sterilised substrate in open air to cool down near to room temperature, thus making the substrate ready for inoculation.

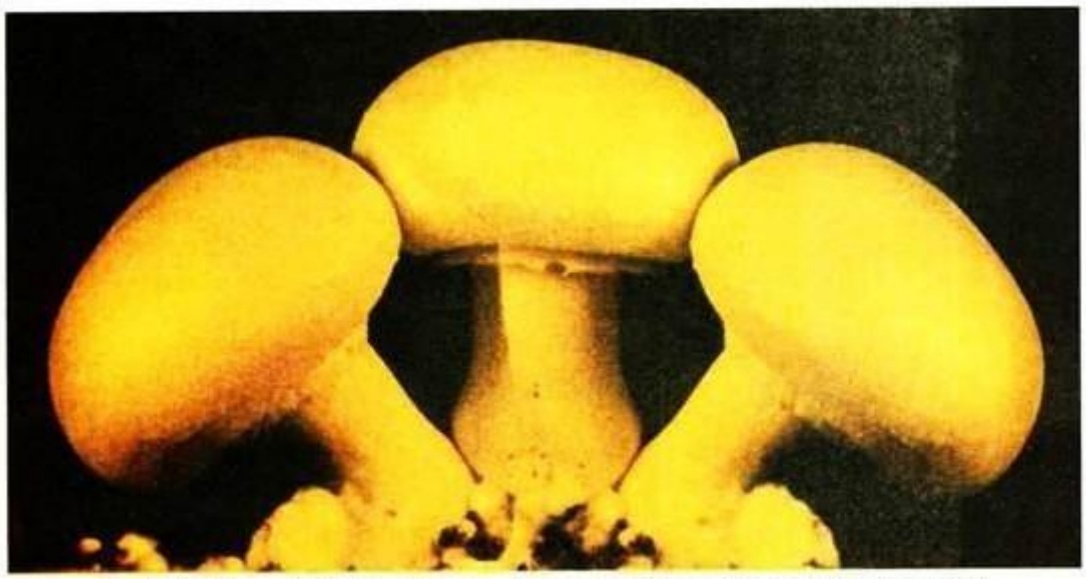


Fig. 4.108 : Mature fruit bodies of *Agaricus brunnescens* (syn. *A. bisporus*) on mushroom bed
[Courtesy : Dr Samir Datta]

Inoculation of Substrate:

The substrate is then inoculated with the mycelial culture (developed earlier, either in Potato Dextrose Agar i.e., PDA or Yeast Potato Dextrose Agar i.e., YPDA or Malt extract Agar and Rice bran decoction medium).

Incubation:

Incubate the inoculated container at 20-25°C in dark for 3 weeks. Shake the container after a few days, when the mycelial growth becomes visible on the grain.

Storage of Spawn:

Store the spawn at 0-4°C in a refrigerator for a maximum period of 6 months, if it is not needed immediately.

The spawn can be purchased from any spawn-growing centre. (The spawn is also available in "National Centre for Mushroom Research and Training (NCMRT)", Chambaghat, Solan 173 213, Himachal Pradesh, India.

2. Preparation of Compost:

The compost used in the cultivation are of two types:

Natural and Synthetic:

i. Natural Compost:

The natural compost is prepared by mixing barley or wheat straw with fresh and pure horse dung (not with the dung of other animal). Mixed, rain wet or old dung is not suitable for the

preparation of compost. Commonly 100 kg of dung is mixed with 33 kg of straw. The mixture is then stacked a metre high heap.

The heap of mixture should be kept under shade in open air. After 3-4 days, the heap was turned (to release ammonia) and stacked again. The turning process is repeated 4-5 times at an interval of 5-6 days. During this process, gypsum ($CaSO_4.2H_2O$) is added @ 25 kg/tonne (1,000 kg) dung. Finally, 40 ml nemagon is sprayed and added to the mixture. The compost was then filled in the tray of 100 x 50 x 15 cm size.

ii. Synthetic Compost:

The ingredients required for the synthetic compost are:

(a) Chopped wheat straw (3-6 cm size) 300 kg

(b) Wheat bran 30 kg

(c) Calcium ammonium nitrate or Ammonium sulphate 6 kg

(d) Urea 4 kg

(e) Potash 1.5 kg

(f) Calcium sulphate (gypsum) 30 kg

(g) Sawdust 10 kg

Wet the sawdust with water by spraying and mix half of the ingredient, except wheat straw and gypsum. Next day, spread the wheat straw on the cement floor and wet it thoroughly by spraying with water. The sawdust-chemical mixture is then mixed thoroughly with wetted wheat straw. This mixture is then stacked under shade into a metre high heap and covered with polythene sheet.

After 5 days, the stack is scraped and rest half ingredient is thoroughly mixed with it and the entire mixture is then stacked again. This process is repeated six times. Calcium sulphate is added in the 3rd and 4th turning.

Normally the compost becomes ready to cultivate after 6th turning, but 2 or more turning may be given if the smell of ammonia is yet there in the compost. During last turning, insecticide like malathion (10 ml dissolve in 5 I water) is added to the prepared compost. The prepared compost will be brown or dark brown in colour and is sufficient enough to fill 25 trays of 100 x 50 x 15 cm size.

3. Filling of Trays with Compost:

Mix 3 kg of calcium carbonate with the compost prepared earlier. Fill the wooden trays with compost and compress fairly by using a wooden board (1 2 cm x 25 cm), so that a space of about 3 cm deep is left on the top of the tray.

4. Spawning i.e., Inoculation of Compost:

Spread the spawn on the surface of compost and then cover by a thin layer of compost. Little pressure with the fingers is given to make good contact of spawn with compost. Finally the trays are covered with old newspaper. The trays are arranged one after the other in vertical stacks in such a way that sufficient aeration between the trays is maintained.

5. Watering of Inoculated Compost Filled Trays:

Sprinkled water to be given on newspaper to maintain humidity. Water should be applied twice a day or less depending on the availability of moisture. The room temperature should be maintained between 24°C and 25°C for 12-15 days for the good growth of mycelium on the compost. The mycelium appears in the form of white cottony growth on the surface of bed.

6. Casing:

The process of covering the mycelial mat on compost, surface is made with a thin layer of soil mixed with different substances.

The casing can be done with different types of mixture like:

i. Soil : Sand : : 1 : 1;

ii. Well-rotten cow dung: light soil: : 3 : 1 ;

iii. Spent compost: Sand: Slaked lime : : 4:1:1 etc.

Casing soil should be sterilised either by chemicals like methyl bromide, formalin etc. or by heating at 70-75°C temperature for 6 hours to kill the inhabiting fungi, nematodes, insects etc.

The fruit bodies of mushroom are expected to appear after 5-20 days of casing. After casing, the room temperature should be maintained between 14-18°C for the good growth of the fruit body. The fruit bodies attain the size of button stage from pinhead within 7-8 days. Next crop appears at an interval of 8-10 days.

7. Harvesting of Mushrooms i.e., Fruit Bodies:

When the cap of the fruit body is tight with its stalk, the fruit bodies are harvested. The fruit bodies are harvested by twisting and uprooting, after holding the basal region of stalk with fingers. The lower part of the stalk is cut out where the compost remains attached.

8. Storage of Mushrooms:

The fruit bodies may be stored at 4°C for a few days, if it is not consumed or marketed immediately.

B. Cultivation of Paddy Straw Mushroom (Volvariella Volvacea):

The paddy straw mushroom is also called tropical, straw or Chinese mushroom (Fig. 4.109). In West Bengal, it is called as 'Poal chatu'. The genus Volvariella belongs to the family Pluteaceae under the order Agaricales of Basidiomycotina.

The common edible species under this genus are V. volvacea, V. diplasia and K esculenta; those are grown commercially in different countries like Burma (Myanmar), China, Philippines, Malaya, India etc.

In addition to paddy straw, other substrates like water hyacinth, cotton waste, banana leaves, sawdust, sugarcane thrash (bagasse) etc., are used as substrate due to the presence cellulose, hemicellulose and lignin.

In India, the cultivation of this mushroom was first initiated in Coimbatore, Tamil Nadu, and now it is popular in different tropical parts due to the requirement of temperature ranges between 30-45°C.

The process of cultivation of straw mushroom is as follows:

1. Requirements,
2. Preparation of spawn,
3. Cultivation procedure,
4. Harvesting of fruit bodies, and
5. Preservation of fruit bodies.

1. Requirements:

i. Spawn of V. volvacea (600-800 gms grain spawn/bed),

ii. Bricks,

iii. Bamboo frame (1 m x 1 m),

iv. Small water tank,

v. Paddy straw (preferably from aman variety), apx. 36 kg,

vi. Loose straw 5-6 kg,

vii. Powder of Gram or Arhar seeds 200-250 gm,

viii. Thermometer (0-100°C scale), and ix. White polythene sheet.

2. Preparation of Spawn:

The spawn can be prepared following the same procedure as adopted in Agaricus brunnescens (see page 395). But in addition to grains of wheat or sorghum, the rice straw can also be used as substrate.

3. Cultivation Procedure:

Fresh paddy straw, not more than one year old and preferably from the Aman variety, should be collected from farmer or from any store. 24 straw bundles of about 1.5 kg each along with some loose straw are immersed completely in a water-filled tank by putting some weight on the bundles for about 12-15 hours.

Then take out the straw bundles from the tank and keep them in stack on cement floor to drain off excess water.

Fig. 4.109 : Mature fruit bodies of *Volvariella volvacea*

[Courtesy : Dr. Samir Datta]

4. Preparation of Bed and Spawning:

One square bed of 1 m x 1 m x 1 m or 1 m x 0.75 m x 1 m is prepared with pre-soaked straw, keeping the butt ends (basal region) at one side, placed close to each other and arranged

length-wise on a bamboo frame, supported on 4 pillars made of bricks. Same number of soaked straw bundles are placed on the previous one by keeping the butt ends in opposite direction.

Inoculate the bed with spawn. The bids of spawn are placed about 8-10 cm inside the margin, maintaining a space of about 5 cm from each other. About 160-200 grams spawn is required for each layer. Powders of Cram or Arhar seeds of about 50 gms or more are spread along the line of spawning.

Second and third layers are arranged and inoculated in a similar process, but 2nd layer is placed at right angle to the 1st layer and the third layer is like the 1st layer. The spawn and seed powder on the 2nd layer will be given like the 1st layer, but on the 3rd layer those will be distributed uniformly throughout the bed.

Finally, cover the top layer with loose straw. Loosely bind the bed with rope made of wheat straw at the three regions, one in the middle and one on each side. Press the bed with the help of wooden board to release the internal air and thus the spawn get compressed with the wet straw bundles. Cover the bed with polythene sheet.

Watering should be done once or twice with the help of micro-sprayer. The temperature of the bed should remain 30-35°C after spawning and it should not go below 30°C during the growing season. The relative humidity should be between 80-90%.

Polythene sheet should be removed after 7-10 days of spawning for the appearance of button of the mushroom. After that the buttons quickly develop into fruit bodies.

The straw once used in the mushroom cultivation can be used again. The bed should be prepared under shade away from direct sunlight and rain and also in well-aerated condition, but wind should not blow very fast.

5. Harvesting of Mushroom:

The fruit bodies are harvested by gentle twisting when the volva is about to rupture or is just ruptured. The production continues for 25- 30 days, but in two phases. The total production per bed is approximately 3 kg. The production of second phase is comparatively less.

6. Preservation:

The fruit bodies are consumed fresh or can be preserved by drying or in refrigerator for 27-48 hours. Drying can be done either in the sun or in oven at 50-60°C temperature.

C. Cultivation of Oyster Mushroom (Pleurotus):

Species of Pleurotus are commonly called Oyster mushroom or Dhingri or Wood fungus (Fig. 4.110). It is the fourth important mushroom in the world ranking with an annual production of about 15,000 tones. It grows commercially in Japan, Taiwan, Italy, France, Thailand, Philippines and India, out of which the first three are the leading countries in its production.

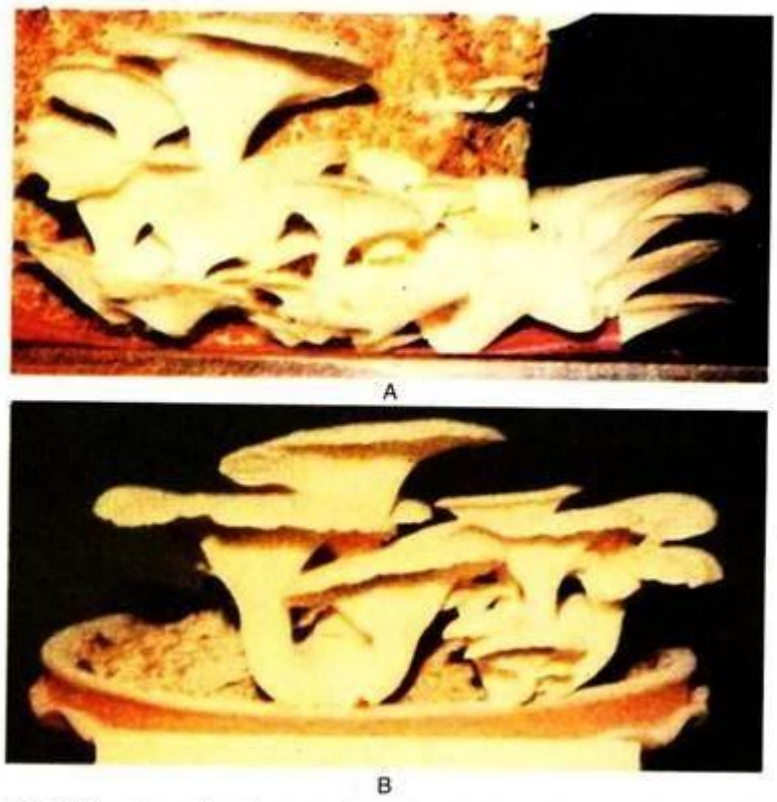


Fig. 4.110 : Fruit bodies of *Pleurotus sajor-caju* on mushroom bed : A. Mushroom bed prepared in polythene bag and B. Mushroom bed prepared on earthern pot

[Courtesy : Dr. Samir Datta]

The genus Pleurotus contains more than 50 species, of which P. flabellatus, P. ostreatus, P. sajor-caju, P. sapidus, P. fossulatus, P. cornu- copieae, P. sapathulatus and P. florida have been cultivated in India.

The process of cultivation of Oyster mushroom is as follows:

1. Requirements,
2. Preparation of spawn,
3. Cultivation procedure,
4. Harvesting of fruit bodies,
5. Preservation of fruit bodies.

Requirements:

i. Spawn of Pleurotus – 60 gms (30 gms grain spawn/kg of paddy straw),

ii. Chopped and dry paddy straw (1-2 cm) -2 kg,

iii. Gunny or polythene bag (40″ x 24″) – 1 piece.

iv. Horse gram powder – 50 gms (25 gm.s/kg),

v. Thick polythene sheet (5 ft x 5 ft) – 1 piece,

vi. Polythene bags (30″ x 18″) – 2 piece, and

vii. Water sprayer. Preparation of spawn

The spawn can be prepared as per method adopted in Agaricus brunnescens (see page 395). The spawn should not be older than 1 month.

Cultivation Procedure:

Take two kg of chopped straw (preferably of Aman variety) in a gunny or incised polythene bag and tighten the mouth with rope. Immerse the bag completely in water (90 I water containing 7 gm Bavistin, a fungicide along with 125 ml formaldehyde) filled tank by putting some weight on it for approximately 12-15 hours.

Then take out the straw bags from the tank and keep the straw pieces in a wicker basket or a scuttle (Beng. Jhuri). Put more water in the wet straw to remove dirt, rags etc. Wait for one or more hours to drain off excess water.

The wet straw pieces are then kept on polythene sheet and mixed with powder of Horse gram (20-25 gms/kg) and spawn (30 gms/kg) and if possible 10 gms fertiliser of IFCO or P.P.L (10:26: 26) may be added.

Take 2 polythene bags (appx. 30″ x 18″) and make 6-12 holes at the lower side of each bag. Then the entire mixture is put equally inside the two bags. Keep the filled bags on a bench or table in a room at 21-30°C and 65-80% humidity, with sufficient light and ventilation for 15-16 days, for spawn running.

Spray water on bed twice a day by micro-sprayer. After 15-16 days, the straw pieces are covered with the mycelium and form a solid cylindrical mass. Remove the polythene bag and keep the mass on the same polythene bag in the same place. The compact mass should be watered 4-8 times throughout the day with the micro-sprayer.

The young fruit bodies will be developed after 3-4 days (i.e., 18-20 days of spawning) from all sides of the bed.

Within 2-3 days, the fruit bodies attain the size of harvesting. After harvesting the straw-mycelium mass again put inside the bag and tighten the-mouth with rope. Keep it for 7 days and then again remove the mass from polythene bag and keep the polythene bag as before.

Next crop of mushroom will be available within 7 days i.e., approximately 36 days after starting. Repeat the process again and the third crop will be available in 50 days. During cropping period light should be provided for 15-20 minutes/day for better yield.

Harvesting of Mushroom:

The fruit bodies are harvested by gentle twisting after holding the base of the fruit bodies with fingers. The fruit bodies can be harvested generally 3 times i.e., at 22, 36 and 50 days and the total production will be 2 kg. Afterwards the bed should be destroyed.

Preservation:

After harvesting, the fresh mushrooms can be sold in the market or they can be dried in sun (for three consecutive days) or in oven at 65°C. The cultivation of Pleurotus can also be done on earthen tray or tub.

Other Modified Procedure:

Like the above procedure, the mixture is prepared and the tray or tub is filled with the mixture. The open end should be covered with polythene sheet. Watering should be done twice a day (varies with requirement) with the micro- sprayer.

The mycelium will spread throughout the sub-stratum within 10-12 days. The fruit bodies will start to develop after 20-24 clays of spawning. Within 2-3 days the fruit bodies will be the size of harvesting.

Some Edible and Poisonous Mushrooms:

Both edible and poisonous mushrooms are available in nature. List of some edible and poisonous mushrooms are given below. Common name of mushrooms are given in parenthesis.

A. Edible Mushrooms:

a. Members of Basidiomycotina:

1. Agaricus brunnescens (white button),
2. A. campestris (field mushroom),
3. Pleurotus edodes (shiitake),
4. P. sajor-caju (oyster),

5. Volvariella volvacea (paddy straw) etc.

b. Members of Ascomycotina:

1. Morchella conica,

2. M. esculenta.

B. Poisonous Mushrooms:

1. Amanita phalloides (death cap): Toxic principles are α and β-amanitin, and phalloidin.

2. A. virosa (destroying angles),

3. A. verna (fool's cap),

4. A. muscaria (fly-agaric) etc.

Diseases of Mushrooms:

Like higher plants, mushrooms also suffer from different diseases caused by fungi, bacteria and viruses.

List of some diseases are given in Table 4.20:

Table. 4.20 : List of some diseases of mushrooms

Name of diseases	Causal organisms
A. Fungal diseases	
1. Wet bubble disease	*Mycogone perniciosa*
2. Dry bubble disease	*Vertillium fungicola* var. *fungicola*, *V. fungicola* var. *aleophilum*
3. Soft mildew or Cobweb	*Hypomyces rosellus*
4. False truffle	*Diehliomyces micxrosporus* syn. *Pseudobalsamia microspore*
5. Shaggy stipe	*Mortierella bainieri*
6. Gill mildew	*Cephalosporium* spp.
7. Cap spotting	*Aphanocladium album*, etc.
B. Bacterial diseases	
1. Bacterial blotch or brown blotch	*Pseudomonas tolaasi*
2. Ginger blotch	*P. gingeri*
3. Drippy gill	*P. agarici*
C. Viral diseases	
1. Dieback disease	
2. X-disease	
3. Brown disease	
4. Water stripe disease	

Other Organisms which Hamper Mushroom Yield:

In addition to diseases, mushroom yield is also hampered by molds and animal pests.

Molds:

Different types of molds are associated with mushroom growing. Molds are considered as competitors for nutrients or antagonists rather than parasites.

Name of different molds associated with mushroom cultivation is given:

1. Olive-green mold (Chaetomium olivaceum),
2. Green mold (Trichoderma viride),
3. Fire mold (Neurospora crassa),
4. Red geotrichum or lipstick mold (Sporen-donema purpurescens),
5. Sepedonium yellow mold (Sepedonium spp.),
6. White plaster mold (Scopulariopsis fimicola),
7. Brown plaster mold (Papulospora byssina),
8. Black whisker mold (Doratomyces stemonites),
9. Inky cap (Coprinus lagopus and other spp.),
10. Cinnamon mold (Peziza ostracoderma, syn. Plicaria fulva) etc.

Animal Pests:

Like molds, different animal pests also hamper the yield and quality of mushrooms.

These are:

Major Pests:

1. Mushroom flies: Sciarids, Lycoriella solani, L. auripila,
2. Phorids: Megaselia halterata,
3. Phorids: M. nigra,
4. Cecids: Heteropeza pygmaea, Mycophila speyeri and M. barnesi,
5. Tarsonemid mite: Tarsonemus myceliophagus,
6. Red peppes: Pygmephorus spp.
7. Predatory mites: Parasitus fimetorum, Digamasellus fallax, Arctoseius cetratus,
8. Mycophagous eelworms: Ditylenchus myceliophagus and Aphelenchoides composticola.

Minor Pests:

Collembola: Archorutes armatus

Sphaeroceridae: Leptocera heteroneura

Drosophilidae: Drosophila tunebns etc.

CHEESE PRODUCTION

Making cheese is both an art and a science. Cheesemakers rely as much on measurements of pH levels and inoculations of specific molds as they do their own senses of sight, touch, and smell.

There are six important steps in cheesemaking: acidification, coagulation, separating curds and whey, salting, shaping, and ripening. While the recipes for all cheeses vary, these steps outline the basic process of turning milk into cheese and are also used to make cheese at home.

Acidification

The first step to making cheese is acidification. During this stage, a starter culture is added to milk that will change lactose (milk sugar) into lactic acid. This changes the acidity level of the milk and begins the process of turning milk from a liquid into a solid.

Coagulation

Coagulation is the process of transforming the liquid into a semisolid. When making cheese, an enzyme called rennet is added either as a liquid or paste to further encourage the milk to solidify.

Curds and Whey

As the milk solidifies, it forms curd and whey. The curds are the solid part and whey is the liquid. In this step, the curds are cut using a knife or a tool that resembles a rake.

Cutting the curds further encourages them to expel whey. Generally, the smaller the curds are cut, the harder the resulting cheese will be. Soft cheeses like Camembert or Brie are hardly cut at all. Harder cheeses like cheddar and Gruyere are cut into a very fine texture. For these harder cheeses, the curds are further manipulated by cheddaring and/or cooking. Cooking the curd changes its texture, making it tender rather thancrumbly.

When this process is complete, the whey is drained away, leaving the curd alone to become cheese.

Salting

Salt is added for flavor. It also acts as a preservative so the cheese does not spoil during the long months or years it spends aging and it helps to form a natural rind on the cheese.

There are several ways to use salt. Salt can be added directly into the curd as the cheese is being made. The outside of the wheel of cheese can be rubbed with salt or with a damp cloth that has

been soaked in brine (heavily salted water). The cheese can also be bathed directly in a vat of brine, as it is for mozzarella.

Shaping

In this stage, each type of cheese takes its familiar form as a solid block or wheel. The cheese is put into a basket or a mold to form it into a specific shape. At the same time, the cheese is also pressed with weights or a machine to expel any remaining liquid.

Ripening

Referred to as *affinage*, this process ages cheese until it reaches optimal ripeness. During this time, the temperature and humidity of the cave or room where the cheese ages are closely monitored.

An experienced *affineur* knows how to properly treat each cheese so it develops the desired flavor and texture. For some cheeses, ambient molds in the air give the cheese a distinct flavor. For others, mold is introduced by spraying it on the cheese (Brie) or injecting it into the cheese (blue cheese). Some cheeses must be turned, some must be brushed with oil, and some must be washed with brine or alcohol.

The amount of time a cheese is left to ripen depends on the type of cheese and the cheesemaker's desired outcome. It may take several months to quite a few years for a cheese to age, but once finished, it is ready to be packaged.

BIODIESEL

Biodiesel is a form of diesel fuel derived from plants or animals and consisting of long-chain fatty acid esters. It is typically made by chemically reacting lipids such as animal fat (tallow),soybean oil or some other vegetable oil with an alcohol, producing a methyl, ethyl or propyl ester.

Unlike the vegetable and waste oils used to fuel converted diesel engines, biodiesel is a drop-in biofuel, meaning it is compatible with existing diesel engines and distribution infrastructure. Biodiesel can be used alone or blended with petrodiesel in any proportions. Biodiesel blends can also be used as heating oil.

Production:

Biodiesel is commonly produced by the transesterification of the vegetable oil or animal fat feedstock, and other non-edible raw materials such as frying oil, etc. There are several methods for carrying out this transesterification reaction including the common batch process, heterogeneous catalysts, supercritical processes, ultrasonic methods, and even microwave methods.

Chemically, transesterified biodiesel comprises a mix of mono-alkyl esters of long chain fatty acids. The most common form uses methanol (converted to sodium methoxide) to produce methyl esters (commonly referred to as Fatty Acid Methyl Ester – FAME) as it is the cheapest alcohol available, though ethanol can be used to produce an ethyl ester (commonly referred to as Fatty Acid Ethyl Ester – FAEE) biodiesel and higher alcohols such as isopropanol and butanol have also been used. Using alcohols of higher molecular weights improves the cold flow properties of the resulting ester, at the cost of a less efficient transesterification reaction. A lipid transesterification production process is used to convert the base oil to the desired esters. Any free fatty acids (FFAs) in the base oil are either converted to soap and removed from the process, or they are esterified (yielding more biodiesel) using an acidic catalyst. After this processing, unlike straight vegetable oil, biodiesel has combustion properties very similar to those of petroleum diesel, and can replace it in most current uses.

The methanol used in most biodiesel production processes is made using fossil fuel inputs. However, there are sources of renewable methanol made using carbon dioxide or biomass as feedstock, making their production processes free of fossil fuels.

Biodiesel feedstocks

A variety of oils can be used to produce biodiesel. These include:

- Virgin oil feedstock – rapeseed and soybean oils are most commonly used, soybean oil [3] accounting for about half of U.S. production. It also can be obtained from Pongamia, field pennycress and jatropha and other crops such as mustard, jojoba, flax, sunflower, palm oil, coconut and hemp (see list of vegetable oils for biofuel for more information);
- Waste vegetable oil (WVO);
- Animal fats including tallow, lard, yellow grease, chicken fat, and the by-products of the production of Omega-3 fatty acids from fish oil.
- Algae, which can be grown using waste materials such as sewageand without displacing land currently used for food production.
- Oil from halophytes such as *Salicornia bigelovii*, which can be grown using saltwater in coastal areas where conventional crops cannot be grown, with yields equal to the yields of soybeans and other oilseeds grown using freshwater irrigation
- Sewage Sludge – The sewage-to-biofuel field is attracting interest from major companies like Waste Management and startups like InfoSpi, which are betting that renewable sewage biodiesel can become competitive with petroleum diesel on price.

Yield

Feedstock yield efficiency per unit area affects the feasibility of ramping up production to the huge industrial levels required to power a significant percentage of vehicles.

Some typical yields

Crop	Yield	
	L/ha	**US gal/acre**
Palm oil[n 1]	4752	508
Coconut	2151	230
Cyperus esculentus	1628	174
Rapeseed	954	102

Soy (Indiana)	554-922	59.2–98.6
Chinese tallow	907	97
Peanut	842	90
Sunflower	767	82
Hemp	242	26

UNIT V

RECOMBINANT PROTEIN FOR THERAPEUTICAL APPLICATIONS AND DIAGNOSIS

THE PRODUCTION, ADVANTAGES AND LIMITATIONS OF MONOCLONAL ANTIBODIES.

Antibodies or immunoglobulin's are protein molecules produced by a specialized group of cells called B-lymphocytes (plasma cells) in mammals. The structures, characteristics and various other aspects of immunoglobulin's (Igs) are described elsewhere. Antibodies are a part of the defense system to protect the body against the invading foreign substances namely antigens.

Each antigen has specific antigen determinants (epitopes) located on it. The antibodies have complementary determining regions (CDRs) which are mainly responsible for the antibody specificity. In response to an antigen (with several different epitopes), B-lymphocytes gear up and produce many different antibodies. These types of antibodies which can react with the same antigen are designated as polyclonal antibodies.

The polyclonal antibody production is variable and is dependent on factors such as epitopes, response to immunity etc. Due to lack of specificity and heterogenic nature, there are several limitations on the utility of polyclonal antibodies for therapeutic and diagnostic purposes.

Monoclonal antibody (MAb) is a single type of antibody that is directed against a specific antigenic determinant (epitope). It was a dream of scientists to produce MAbs for different antigens. In the early years, animals were immunized against a specific antigen, B-lymphocytes were isolated and cultured in vitro for producing MAbs. This approach was not

successful since culturing normal B-lymphocytes is difficult, and the synthesis of MAb was short-lived and very limited.

It is interesting that immortal monoclonal antibody producing cells do exist in nature. They are found in the patients suffering from a disease called multiple myeloma (a cancer of B-lymphocytes). It was in 1975. George Kohler and Cesar Milstein (Nobel Prize, 1984) achieved large scale production of MAbs. They could successfully hybridize antibody—producing B-lymphocytes with myeloma cells in vitro and create a hybridoma.

The result is that the artificially immortalized B-lymphocytes can multiply indefinitely in vitro and produce MAbs. The hybridoma cells possess the growth and multiplying properties of myeloma cells but secrete antibody of B-lymphocytes. The production of monoclonal antibodies by the hybrid cells is referred to as hybridoma technology.

Principle for Creation of Hybridoma Cells:

The myeloma cells used in hybridoma technology must not be capable of synthesizing their own antibodies. The selection of hybridoma cells is based on inhibiting the nucleotide (consequently the DNA) synthesizing machinery. The mammalian cells can synthesize nucleotides by two pathways—de novo synthesis and salvage pathway (Fig. 17.1).

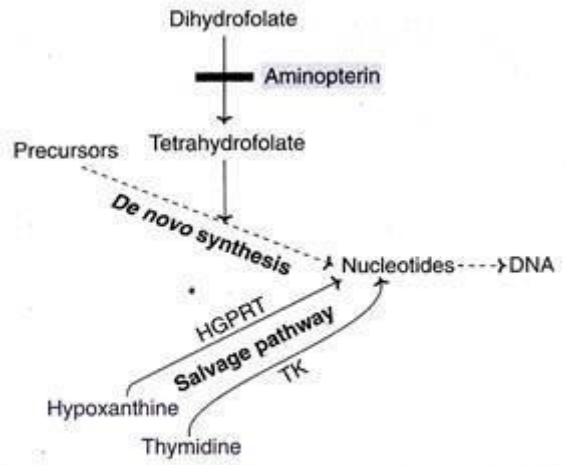


Fig. 17.1 : Pathways for the synthesis of nucleotides (HGPRT–Hypoxanthine guanine phosphoribosyl transferase; TK–Thymidine kinase)

The de novo synthesis of nucleotides requires tetrahydrofolate which is formed from dihydrofolate. The formation of tetrahydrofolate (and therefore nucleotides) can be blocked by the inhibitor aminopterin. The salvage pathway involves the direct conversion of purines

and pyrimidine's into the corresponding nucleotides. Hypoxanthine guanine phosphoribosyl transferase (HGPRT) is a key enzyme in the salvage pathway of purines.

It converts hypoxanthine and guanine respectively to inosine monophosphate and guanosine monophosphate. Thymidine kinase (TK), involved in the salvage pathway of pyrimidine's converts thymidine to thymidine monophosphate (TMP). Any mutation in either one of the enzymes (HGPRT or TK) blocks the salvage pathway.

When cells deficient (mutated cells) in HGPRT are grown in a medium containing hypoxanthine aminopterin and Thymidine (HAT medium), they cannot survive due to inhibition of de novo synthesis of purine nucleotides (Note : Salvage pathway is not operative due to lack of HGPRT). Thus, cells lacking HGPRT, grown in HAT medium die.

The hybridoma cells possess the ability of myeloma cells to grow in vitro with a functional HGPRT gene obtained from lymphocytes (with which myeloma cells are fused). Thus, only the hybridoma cells can proliferate in HAT medium, and this procedure is successfully used for their selection.

Production of Monoclonal Antibodies:

The establishment of hybridomas and production of MAbs involves the following steps (Fig. 17.2).

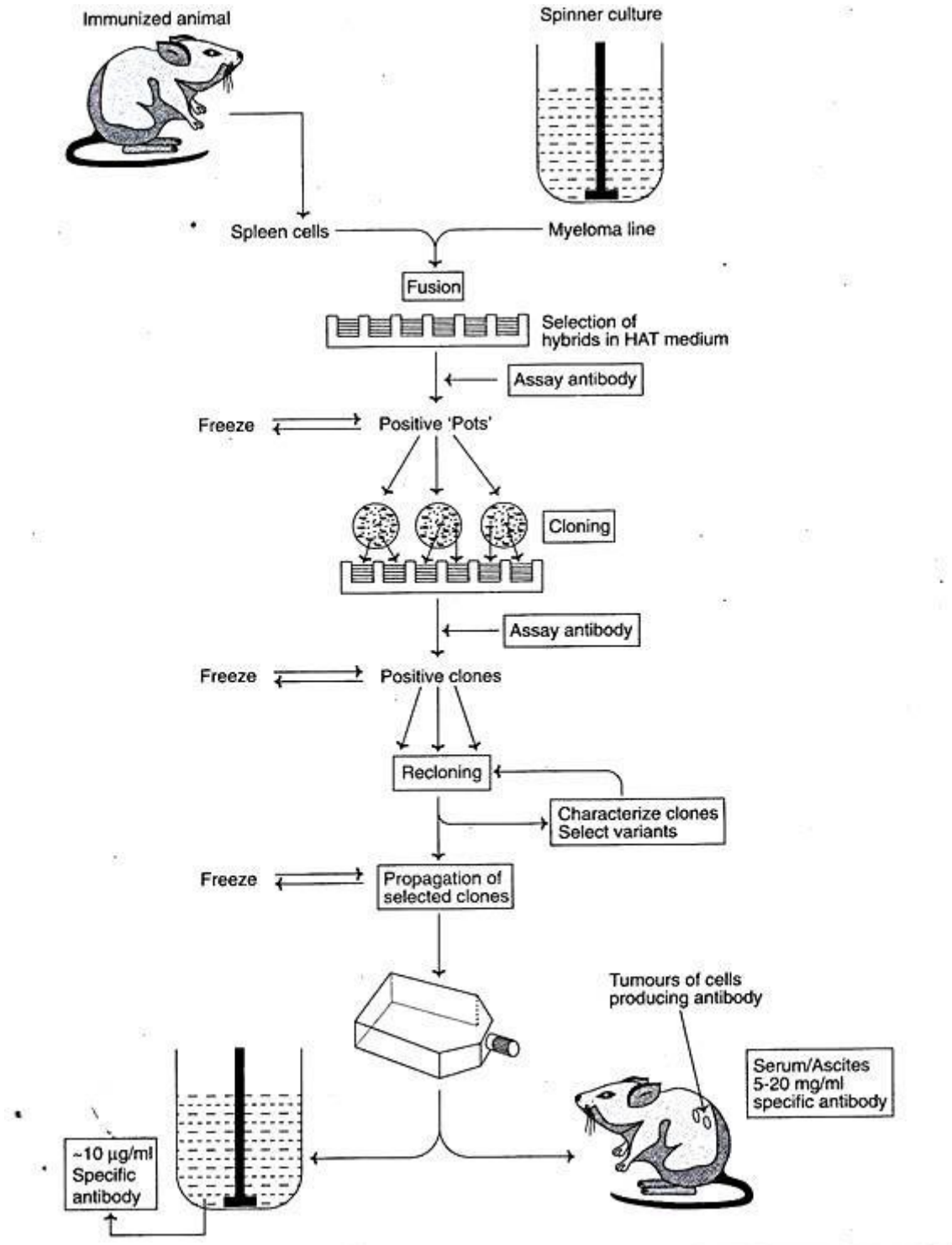


Fig. 17.2 : Basic protocol for the derivation of monoclonal antibodies from hybrid myelomas.

1. Immunization

2. Cell fusion
3. Selection of hybridomas
4. Screening the products
5. Cloning and propagation
6. Characterization and storage.

1. Immunization:

The very first step in hybridoma technology is to immunize an animal (usually a mouse), with appropriate antigen. The antigen, along with an adjuvant like Freund's complete or incomplete adjuvant is injected subcutaneously (adjuvants are non-specific potentiators of specific immune responses). The injections at multiple sites are repeated several times.

This enables increased stimulation of B-lymphocytes which are responding to the antigen. Three days prior to killing of the animal, a final dose of antigen is intravenously administered. The immune-stimulated cells for synthesis of antibodies have grown maximally by this approach. The concentration of the desired antibodies is assayed in the serum of the animal at frequent intervals during the course of immunization.

When the serum concentration of the antibodies is optimal, the animal is sacrificed. The spleen is aseptically removed and disrupted by mechanical or enzymatic methods to release the cells. The lymphocytes of the spleen are separated from the rest of the cells by density gradient centrifugation.

2. Cell Fusion:

The thoroughly washed lymphocytes are mixed with HGPRT defective myeloma cells. The mixture of cells is exposed to polyethylene glycol (PEG) for a short period (a few minutes), since it is toxic. PEG is removed by washing and the cells are kept in a fresh medium. These cells are composed of a mixture of hybridomas (fused cells), free myeloma cells and free lymphocytes.

3. Selection of Hybridomas:

When the cells are cultured in HAT medium (the principle described above), only the hybridoma cells grow, while the rest will slowly disappear. This happens in 7-10 days of culture. Selection of a single antibody producing hybrid cells is very important. This is possible if the hybridomas are isolated and grown individually. The suspension of hybridoma

cells is so diluted that the individual aliquots contain on an average one cell each. These cells, when grown in a regular culture medium, produce the desired antibody.

4. Screening the Products:

The hybridomas must be screened for the secretion of the antibody of desired specificity. The culture medium from each hybridoma culture is periodically tested for the desired antibody specificity. The two techniques namely ELISA and RIA are commonly used for this purpose. In both the assays, the antibody binds to the specific antigen (usually coated to plastic plates) and the unbound antibody and other components of the medium can be washed off. Thus, the hybridoma cells producing the desired antibody can be identified by screening. The antibody secreted by the hybrid cells is referred to as monoclonal antibody.

5. Cloning and Propagation:

The single hybrid cells producing the desired antibody are isolated and cloned. Two techniques are commonly employed for cloning hybrid cells-limiting dilution method and soft agar method.

Limiting dilution method:

In this procedure, the suspension of hybridoma cells is serially diluted and the aliquots of each dilution are put into micro culture wells. The dilutions are so made that each aliquot in a well contains only a single hybrid cell. This ensures that the antibody produced is monoclonal.

Soft agar method:

In this technique, the hybridoma cells are cultured in soft agar. It is possible to simultaneously grow many cells in semisolid medium to form colonies. These colonies will be monoclonal in nature. In actual practice, both the above techniques are combined and used for maximal production of MAbs.

6. Characterization and Storage:

The monoclonal antibody has to be subjected to biochemical and biophysical characterization for the desired specificity. It is also important to elucidate the MAb for the immunoglobulin class or sub-class, the epitope for which it is specific and the number of binding sites it possesses.

The stability of the cell lines and the MAbs are important. The cells (and MAbs) must be characterized for their ability to withstand freezing, and thawing. The desired cell lines are frozen in liquid nitrogen at several stages of cloning and culture.

Large Scale Production of MAbs:

The production MAbs in the culture bottles is rather low (5-10 (ig/ml). The yield can be increased by growing the hybrid cells as ascites in the peritoneal cavity of mice. The ascitic fluid contains about 5-20 mg of MAb/ml. This is far superior than the in vitro cultivation techniques.

But collection of MAb from ascitic fluid is associated with the heavy risk of contamination by pathogenic organisms of the animal. In addition, several animals have to be sacrificed to produce MAb. Hence, many workers prefer in vitro techniques rather than the use of animals.

Encapsulated hybridoma cells for commercial production of MAbs:

The yield of MAb production can be substantially increased by increasing the hybridoma cell density in suspension culture. This can be done by encapsulating the hybridomas in alginate gels and using a coating solution containing poly-lysine (Fig. 17.3). These gels allow the nutrients to enter in and antibodies to come out.

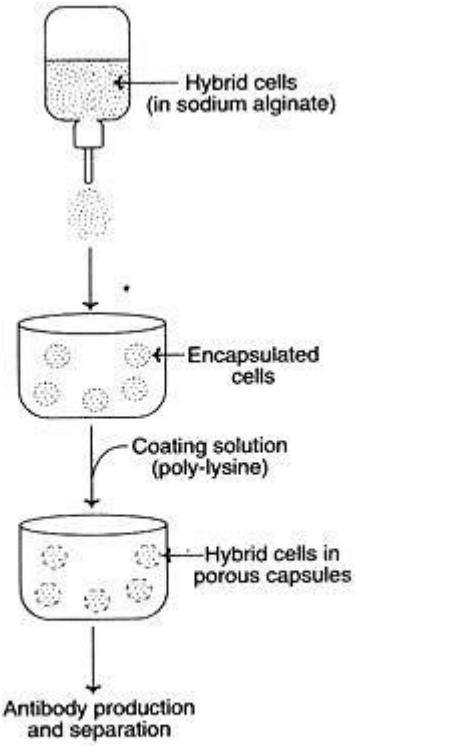


Fig. 17.3 : Production of monoclonal antibodies by microencapsulation.

By this approach, a much higher concentration of MAb production (10-100 µg/ml) can be achieved. Damon Biotech Company and Cell-Tech use encapsulated hybridoma cells for large-scale production of MAbs. They employ 100-liter fermenters to yield about 100g of MAbs in about 2 weeks period.

Human Monoclonal Antibodies:

The monoclonal antibodies produced by using mice are quite suitable for in vitro use. However, their administration to humans is associated with immunological complications, since they are foreign to human body. Production of human monoclonal antibodies is preferred. However, it is difficult to produce human MAbs by conventional hybridoma technology.

The following are the major limitations:

i. For ethical reasons, humans cannot be immunized against antigens.

ii. The fused human lymphocyte-mouse myeloma cells are very unstable.

iii. There are no suitable myeloma cells in humans that can replace mouse myeloma cells.

For the above reasons, alternative arrangements are made to produce human MAbs. These are briefly described below.

Viral transformation of human B-lymphocytes:

B-Lymphocytes, actively synthesizing antibody, are treated with fluorescent-labeled antigen. The fluorescent-activated cells are separated. However, B-cells on their own, cannot grow in culture. This limitation can be overcome by transforming B-lymphocytes with Epstein-Bar virus (EBV). Some of the EBV-transformed cells can grow in culture and produce monoclonal antibodies. Unfortunately, the yield of MAb is very low by this approach.

SCID mouse for producing human MAbs:

The mouse suffering from severe combined immunodeficiency (SCID) disease lacks its natural immunological system. Such mouse can be challenged with appropriate antigens to produce human MAbs.

Transgenic mouse for producing human MAbs:

Attempts have been made in recent years to introduce human immunoglobulin genes into the mice to develop transgenic mice. Such mice are capable of synthesizing human immunoglobulin's when immunized to a particular antigen. The B-lymphocytes isolated from transgenic mice can be used to produce MAbs by the standard hybridoma technology. The above three approaches are quite laborious, and the yield of human MAbs is very low. Consequently, researchers continue their search for better alternatives.

Genetic Engineering Strategies for the Production of Human- Mouse MAbs:

With the advances in genetic engineering, it is now possible to add certain human segments to a mouse antibody. This is truly a hybridized antibody and is referred to as humanized antibody or chimeric antibody.

Substitution of Fv region of human Ig by mouse Fv:

The DNA coding sequences for Fv regions of both L and H chains of human immunoglobulin are replaced by Fv DNA sequence (for L and H chains) from a mouse monoclonal antibody (Fig. 17.4A). The newly developed humanized MAb has Fc region of Ig being human. This stimulates proper immunological response. The chimeric antibodies produced in this manner were found to be effective for the destruction of tumor cells in vitro.

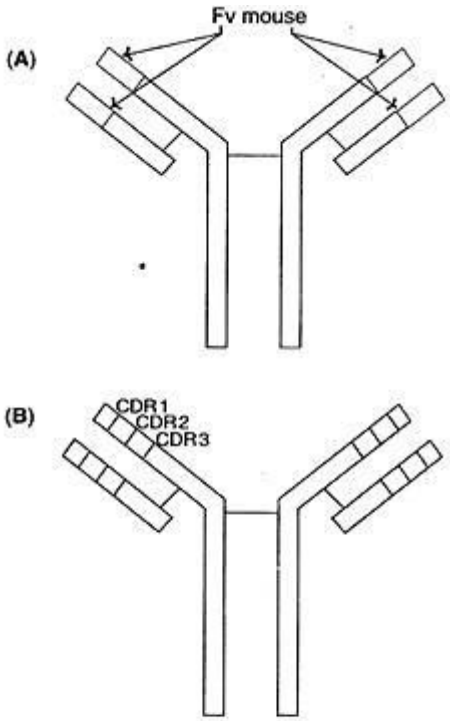


Fig. 17.4 : Genetically engineered human-mouse antibodies (A) Substitution of Fv region of human Ig by mouse Fv (B) Substitution of human Ig by mouse CDRs (CDR-Complementary determining regions).

Substitution of Human Ig by Mouse CDRs:

Genetic engineers have been successful in developing human MAbs containing mouse complementary determining regions (CDRs). This is made possible by replacing CDRs genes (CDR_1, CDR_2, and CDR3) of humans by that of mouse. These chimeric antibodies (Fig. 17.4B) possess the antigen binding affinities of the mouse and they can serve as effective therapeutic agents. So far, about 50 monoclonal antibodies have been produced by this approach. However, this technique is costly and time consuming.

Bi-specific monoclonal antibodies:

The MAbs in which the two arms of Fab (antigen-binding) have two different specificities for two different epitopes are referred to as bi-specific MAbs. They may be produced by fusing two different hybridoma cell lines (Fig. 17.5) or by genetic engineering. Bi-specific Fab MAbs theoretically, are useful for a simultaneous and combined treatment of two different diseases.

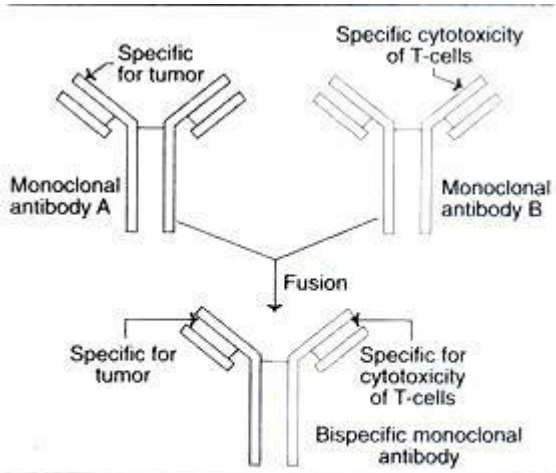


Fig. 17.5 : Production of a bispecific monoclonal antibody.

Production of Mabs in E. coli:

The hybridoma technology is very laborious, expensive and time consuming. To overcome these limitation, researchers have been trying to genetically engineer bacteria, plants and animals. The objective is to develop bioreactors for the large scale production of monoclonal antibodies.

It may be noted that the antigen binding regions of antibody (Fv or Fab fragments) are very crucial, while the Fc portion is dispensable. A schematic representation of the procedure adopted for the production of functional antibody fragments is shown in Fig. 17.6, and is briefly described.

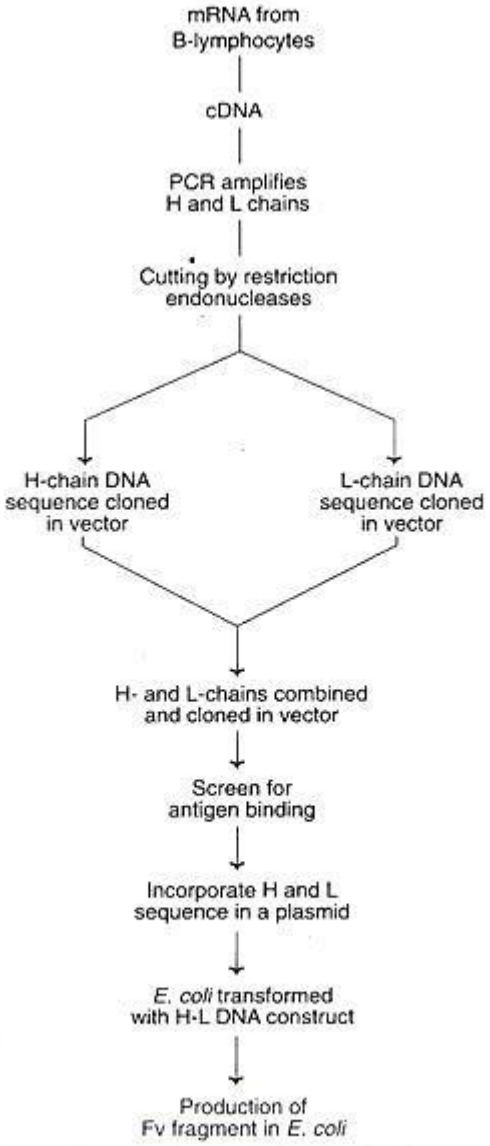


Fig. 17.6 : Production of monoclonal antibodies in E. coli.

The mRNA from isolated B-lymphocytes of either human or mouse is converted to cDNA. The H and L chain sequences of this cDNA are amplified by PCR. The so produced cDNAs are then cut by restriction endonucleases. H and L chain sequences are separately cloned in bacteriophage vectors.

These sequences are put together and cloned in another bacteriophage vector. The combined H and L chains (forming Fv fragment) are screened for antigen binding activity. The specific H and L chains forming a part of the plasmid are transformed in E. coli. These E. coli, in turn, can be harvested to produce Fv fragments to bind to specific antigens.

Second Generation Monoclonal Antibodies:

In the recent years, a number of improvements have been made to produce more specific, sensitive and desired MAbs. This has been possible due to the rapid advances made in genetic engineering techniques. For instances, by employing site- directed mutagenesis, it is possible to introduce cysteine residues at the predetermined positions on the MAb. These cysteine residues which facilitate the isotope labeling may be more useful in diagnostic imaging and radio-immunotherapy.

Advantages of Monoclonal Antibodies:

Monoclonal antibodies truly represent a homogeneous state of a single molecular species. Each MAb is specific to a given antigenic determinant. This is in contrast to the conventional antiserum that contains polyclonal antibodies. The wide range of applications of MAbs is described later.

Limitations of Monoclonal Antibodies:

Hybridoma technology is laborious and time consuming. MAbs are produced against a single antigenic determinant; therefore, they cannot differentiate the molecule as a whole. Sometimes, they may be incapable of distinguishing groups of different molecules also.

The presence of retroviruses as a part of the mammalian chromosomes is a common occurrence. Mice used in MAb production carry several viruses (adenovirus, hepatic virus, retrovirus, reovirus, cytomegalovirus, thymic virus). The presence of some of these viruses has been detected in the hybridomas.

This poses a great danger, since there is no guarantee that MAb produced is totally virus-free, despite the purification. For this reason, US Food and Drug Administration insists that MAb for human use should be totally free from all pathogenic organisms, including viruses.

Vaccine, suspension of weakened, killed, or fragmented microorganisms or toxins or of antibodies or lymphocytes that is administered primarily to prevent disease.

A vaccine can confer active immunity against a specific harmful agent by stimulating the immune system to attack the agent. Once stimulated by a vaccine, the antibody-producing cells, called B lymphocytes, remain sensitized and ready to respond to the agent should it ever gain entry to the body. A vaccine may also confer passive immunity by providing antibodies or lymphocytes already made by an animal or human donor. Vaccines are usually administered by injection (parenteral administration), but some are given orally. Vaccines applied to mucosal surfaces, such as those lining the gut or nasal passages, seem to stimulate a greater antibody response and may be the most-effective route of administration. (For further information, *see* immunization.)

The First Vaccines

The first vaccine was introduced by British physician Edward Jenner, who in 1796 used the cowpox virus (vaccinia) to confer protection against smallpox, a related virus, in humans. Prior to that use, however, the principle of vaccination was applied by Asian physicians who gave children dried crusts from the lesions of people suffering from smallpox to protect against the disease. While some developed immunity, others developed the disease. Jenner's contribution was to use a substance similar to, but safer than, smallpox to confer immunity. He thus exploited the relatively rare situation in which immunity to one virus confers protection against another viral disease. In 1881 French microbiologist Louis Pasteur demonstrated immunization against anthrax by injecting sheep with a preparation containing attenuated forms of the bacillus that causes the disease. Four years later he developed a protective suspension against rabies.

Vaccine Effectiveness

After Pasteur's time, a widespread and intensive search for new vaccines was conducted, and vaccines against both bacteria and viruses were produced, as well as vaccines against venoms and other toxins. Through vaccination, smallpox was eradicated worldwide by 1980, and polio cases declined by 99 percent. Other examples of diseases for which

vaccines have been developed include mumps, measles, typhoid fever, cholera, plague, tuberculosis, tularemia, pneumococcal infection, tetanus, influenza, yellow fever, hepatitis A, hepatitis B, some types of encephalitis, and typhus—although some of those vaccines are less than 100 percent effective or are used only in populations at high risk. Vaccines against viruses provide especially important immune protection, since, unlike bacterial infections, viral infections do not respond to antibiotics.

Vaccine Types

The challenge in vaccine development consists in devising a vaccine strong enough to ward off infection without making the individual seriously ill. To that end, researchers have devised different types of vaccines. Weakened, or attenuated, vaccines consist of microorganisms that have lost the ability to cause serious illness but retain the ability to stimulate immunity. They may produce a mild or subclinical form of the disease. Attenuated vaccines include those for measles, mumps, polio (the Sabin vaccine), rubella, and tuberculosis. Inactivated vaccines are those that contain organisms that have been killed or inactivated with heat or chemicals. Inactivated vaccines elicit an immune response, but the response often is less complete than with attenuated vaccines. Because inactivated vaccines are not as effective at fighting infection as those made from attenuated microorganisms, greater quantities of inactivated vaccines are administered. Vaccines against rabies, polio (the Salk vaccine), some forms of influenza, and cholera are made from inactivated microorganisms. Another type of vaccine is a subunit vaccine, which is made from proteins found on the surface of infectious agents. Vaccines for influenza and hepatitis B are of that type. When toxins, the metabolic by-products of infectious organisms, are inactivated to form toxoids, they can be used to stimulate immunity against tetanus, diphtheria, and whooping cough (pertussis).

vaccinationThe basic strategies behind the use of vaccines to prepare the human immune system to deal with harmful pathogens. Adjuvants, such as aluminum, are incorporated into vaccines to hasten the body's immune response

In the late 20th century, advances in laboratory techniques allowed approaches to vaccine development to be refined. Medical researchers could identify the genes of a pathogen (disease-causing microorganism) that encode the protein or proteins that stimulate the immune response to that organism. That allowed the immunity-stimulating proteins (called antigens) to be mass-produced and used in vaccines. It also made it possible to alter pathogens genetically and produce weakened strains of viruses. In that way, harmful proteins from pathogens can be deleted or modified, thus providing a safer and more-effective method by which to manufacture attenuated vaccines.

Recombinant DNA technology has also proven useful in developing vaccines to viruses that cannot be grown successfully or that are inherently dangerous. Genetic material that codes for a desired antigen is inserted into the attenuated form of a large virus, such as the vaccinia virus, which carries the foreign genes "piggyback." The altered virus is injected into an individual to stimulate antibody production to the foreign proteins and thus confer immunity. The approach potentially enables the vaccinia virus to function as a live vaccine against several diseases, once it has received genes derived from the relevant disease-causing microorganisms. A similar procedure can be followed using a modified bacterium, such as *Salmonella typhimurium*, as the carrier of a foreign gene.

Vaccines against human papillomavirus (HPV) are made from viruslike particles (VLPs), which are prepared via recombinant technology. The vaccines do not contain live HPV biological or genetic material and therefore are incapable of causing infection. Two types of HPV vaccines have been developed, including a bivalent HPV vaccine, made using VLPs of HPV types 16 and 18, and a tetravalent vaccine, made with VLPs of HPV types 6, 11, 16, and 18.

Another approach, called naked DNA therapy, involves injecting DNA that encodes a foreign protein into muscle cells. The cells produce the foreign antigen, which stimulates an immune response.

In addition to the development of memory B cells, which are capable of triggering a secondary immune response upon exposure to the pathogen targeted by a vaccine, vaccination is also beneficial at the population level. When a sufficient number of individuals in a population are immune to a disease, as would occur if a large proportion of a population

were vaccinated, herd immunity is achieved. That means that if there is random mixing of individuals within the population, then the pathogen cannot be spread throughout the population. Herd immunity acts by breaking the transmission of infection or by lessening the chances of susceptible individuals coming in contact with a person who is infectious. Herd immunity provides a measure of protection to individuals who are not personally immune to the disease—for instance, individuals who, because of their age or underlying medical conditions, cannot receive vaccines or individuals who received vaccines but remain susceptible. Herd immunity played an important role in the successful eradication of smallpox, and it is vital in preventing the spread of diseases such as polio and measles.

Adverse Reactions

Vaccination carries some risk of reaction, though adverse effects typically are very rare and very mild. The most common reactions to vaccines include redness and soreness around the vaccination site. More severe adverse reactions, such as vomiting, high fever, seizure, brain damage, or death, are possible for some vaccines. Such reactions are exceptionally rare, however—occurring in less than one in a million people for most vaccines. Severe reactions also tend to affect only certain populations, such as persons whose immune systems are compromised by preexisting disease (e.g., HIV/AIDS) or who are undergoing chemotherapy.

Claims have been made that vaccines are responsible for certain adverse health conditions, particularly autism, speech disorders, and inflammatory bowel disease. Some of those claims focused on thimerosal, a mercury-containing compound used as a preservative in vaccines. Some people believed that autism was a form of mercury poisoning, caused specifically by thimerosal in childhood vaccines. Those claims have been discredited. Still, misinformation and fear generated by false claims about associations between autism and vaccines had a significant impact on individuals' perceptions about vaccine safety. In addition, most individuals in countries where vaccination is widespread have never personally experienced vaccine-preventable disease. Thus, the focus of concern for some people shifted from the negative effects of vaccine-preventable disease to the possible negative effects of the vaccines themselves.

Complacency about vaccine-preventable diseases, combined with concerns over the effects of vaccination, led to decreasing levels of vaccination coverage in some areas of the world. As a consequence, not only were individuals susceptible to vaccine-preventable diseases, but, at

population levels, vaccination rates dropped low enough to cause losses of herd immunity, thereby allowing outbreaks of disease. Such outbreaks brought high costs to societies, especially in terms of health and medical care, disability and economic strain, and loss of life. In the 20th century in Japan, England, and Russia, for example, numbers of children vaccinated against whooping cough dropped sufficiently low so as to enable outbreaks of disease that involved thousands of children and resulted in hundreds of deaths.

SECONDARY METABOLITES FROM PLANT CELL CULTURES

The applications of secondary metabolites and the production process of secondary metabolites in plant cultures.

The production process comprises of seven aspects.

The seven aspects are: (1) Selection of cell lines for high yield of secondary metabolites (2) Large scale cultivation of plant cells (3) Medium composition and effect of nutrients (4) Elicitor-induced production of secondary metabolites (5) Effect of environmental factors (6) Biotransformation using plant cell cultures and (7) Secondary metabolite release and analysis.

Secondary Metabolites:

The chemical compounds produced by plants are collectively referred to as phytochemicals. Biotechnologists have special interest in plant tissue culture for the large scale production of commercially important compounds. These include pharmaceuticals, flavours, fragrances, cosmetics, food additives, feed stocks and antimicrobials.

Most of these products are secondary metabolites— chemical compounds that do not participate in metabolism of plants. Thus, secondary metabolites are not directly needed by plants as they do not perform any physiological function (as is the case with primary metabolites such as amino acids, nucleic acids etc.). Although the native plants are capable of producing the secondary metabolites of commercial interest, tissue culture systems are preferred.

The advantages and limitations are listed:

Major Advantages:

1. Compounds can be produced under controlled conditions as per market demands.
2. Culture systems are independent of environmental factors, seasonal variations, pest and microbial diseases and geographical constraints.
3. Cell growth can be controlled to facilitate improved product formation.
4. The quality of the product will be consistent as it is produced by a specific cell line.
5. Recovery of the product will be easy.
6. Plant cultures are particularly useful in case of plants which are difficult or expensive to be grown in the fields.
7. Mutant cell lines can be developed for the production of novel compounds of commercial importance, which are not normally found in plants.

8. Biotransformation reactions (converting specific substrates to valuable products) can be carried out with certain cultured cells.

9. The production control is not at the mercy of political interference.

10. The production time is less and labour costs are minimal.

Considering the advantages listed above, about 25-30% of medicines for human use, and the various chemical materials for industrial purposes are obtained from plant tissue cultures. In general, tissue culture production of natural materials is cheaper compared to synthetic production. However, there are certain limitations associated with tissue cultures.

Limitations/Disadvantages:

1. In general, in vitro production of secondary metabolites is lower when compared to intact plants.

2. Many a times, secondary metabolites are formed in differentiated tissues/organs. In such a case, culture cells which are non-differentiated can produce little.

3. Cultured cells are genetically unstable and may undergo mutation. The production of secondary metabolite may be drastically reduced, as the culture ages.

4. Vigorous stirring is necessary to prevent aggregation of cultured cells. This may often damage the cells.

5. Strict aseptic conditions have to be maintained during culture technique: Any infection to the culture adversely affects product formation.

Why do Plants Produce Secondary Metabolites?

Based on the existing evidence, it is believed that the production of some secondary metabolites is linked to the induction of morphological differentiation.

Consider the following examples:

1. Cardiac glycosides are found in the leaves of Digitalis.

2. Quinine and quinidine are present in the bark of Cinchona.

3. Tropane alkaloids (e.g. atropine) are found in the roots of Atropa.

It appears that as the cells undergo morphological differentiation and maturation during plant growth, some of the cells specialise to produce secondary metabolites. It is also observed that in vitro production of secondary metabolites is much higher from differentiated tissues when compared to non- differentiated or less differentiated tissues.

Applications of Secondary Metabolites:

From the time immemorial, man has been dependent on the plant products, besides the supply of food from plants. These plant products, mostly the secondary metabolites include pharmaceuticals, flavours, perfumes, agrochemicals, insecticides and raw materials for industries. Chemically, the plant products may be alkaloids, terpenoids, glycosides (steroids, phenolics) etc.

THERAPEUTICAL APPLICATION OF ANIMAL CELL CULTURE

There is a widespread concern that extensive use of animals for laboratory experiments is not morally and ethically justifiable. Animal welfare group's world over are increasingly criticising the use of animals. Some research workers these days prefer to utilize animal cell cultures wherever possible for various studies. The major applications of laboratory animal cell cultures are given in Table 33.3, and listed below.

TABLE 33.3 Summary of the applications of animal cell cultures

Category	*Applications*
Intracellular activity	Studies related to cell cycle and differentiation, transcription, translation, energy metabolism, drug metabolism.
Intracellular flux	Studies involving hormonal receptors, metabolites, signal transduction, membrane trafficking.
Cell to cell interaction	Studies dealing with cell adhesion and motility, matrix interaction, morphogenesis, paracrine control, metabolic cooperation.
Environmental interaction	Studies related to drug actions, infections, cytotoxicity, mutagenesis, carcinogenesis.
Genetics	Studies dealing with genetic analysis, transfection, transformation, immortalization, senescence.
Cell products	Wide range of applications of the cellular products formed (Refer ***Table 33.4***) e.g. vaccines, hormones, interferons etc.

i. Studies on intracellular activity e.g. cell cycle and differentiation, metabolisms.

ii. Elucidation of intracellular flux e.g. hormonal receptors, signal transduction.

iii. Studies related to cell to cell interaction e.g. cell adhesion and motility, metabolic cooperation.

iv. Evolution of environmental interactions e.g. cytotoxicity, mutagenesis.

v. Studies dealing with genetics e.g. genetic analysis, immortalization, senescence.

vi. Laboratory production of medical/pharmaceutical compounds for wide range of applications e.g. vaccines, interferon's, hormones.

There are however, several limitations on the use of animal cell cultures. This is mostly due to the differences that exist between the in vivo and in vitro systems, and the validity of the studies conducted in the laboratory.

Medical / Pharmaceutical Products of Animal Cell Cultures:

The most important application of animal cell cultures is the production of a wide range of commercial compounds for medical and pharmaceutical use. A selected list of animal cell culture products of commercial importance is given in Table 33.4.

Table 33.4 Selected examples of animal cell culture products (proteins) of medical/pharmaceutical importance

Product(s)	*Application(s)*
Vaccines	
Polio vaccines	Poliomyelitis prophylaxis
Measles vaccine	Measles prophylaxis
Rabies vaccine	Rabies prophylaxis
Malaria vaccines	Malaria prophylaxis
HIV vaccine	AIDS prophylaxis and treatment
Plasminogen activators	
Tissue-type plasminogen activator	Acute myocardial infarction, pulmonary embolism, deep vein thrombosis, acute stroke.
Urokinase-type plasminogen activator	
Recombinant plasminogen activator	
Interferons	
Interferon-α	Anticancer, immunomodulator
Interferon-β	Anticancer, antiviral
Interferon-γ	Anticancer, immunomodulator
Blood clotting factors	
Factors VII, VIII, IX and X	Hemophilia, as blood clotting agents.
Hormones	
Human growth hormone	Growth retardation in children
Somatotropin	Chronic renal insufficiency
Follicle stimulating hormone	Treatment of infertility
Human chorionic gonadotropin	Treatment of infertility
Monoclonal antibodies	
Anti-lipopolysaccharide	Treatment of sepsis
Human B-cell Lymphomas	Treatment of B-cell lymphoma
Anti-fibrin 99	Diagnosis of blood clot by imaging
Tcm-FAb (breast)	Diagnosis of breast cancer
Others	
Erythropoietin	Antianaemic agent
Interleukin-2	Anticancer, HIV treatment
Tumor necrosis factor	Anticancer
Granulocyte stimulating factor	Anticancer
Carcinoembryonic antigen	Diagnosis and monitoring of cancer patients.

Production of vaccines:

Monkey kidney or chick embryo cells or recently human diploid cells are in use for the production of vaccines. The vaccine manufacture in animal cell cultures is rather complex

with risk of contamination, and safety aspect. For these reasons, production of vaccines by recombinant DNA technology employing bacteria or yeasts is preferred.

Production of high value therapeutics:

Many human proteins with high therapeutic potential are often in short supply e.g. tissue plasminogen activator, clotting factors (VIII and IX), and erythropoietin. There is a major limitation to produce human proteins that undergo post- translational modifications (glycosylation, carboxylation etc.) in bacteria and yeasts.

This is due to the fact that these organisms do not possess the machinery to perform post-translational changes. However, pharmaceutical proteins that do not require post-translational modifications can be produced by bacteria or yeasts e.g. insulin, albumin, growth hormone. Animal cell cultures (particularly mammalian cell cultures) are useful for the production of many pharmaceutically/medically important proteins (Table 33.4).

These include the following:

i. Plasminogen

ii. Interferon

iii. Blood clotting factors

iv. Hormones

v. Monoclonal antibodies

vi. Erythropoietin.

Purification of pharmaceutical products:

As the desired product is produced in the cell culture medium, its purification, isolation and storage (collectively referred to as downstream processing) assumes significance. The final product for therapeutic applications is expected to satisfy the following criteria.

i. The product should have a stable structure with optimal activity.

ii. The product should be free from other biomolecules that may interfere with its activity and/or cause immunological complications.

iii. It should be free from all pathogens including viruses.

Genetic Engineering of Animal Cells and their Applications:

It is now possible to genetically modify the animal (mammalian) cells to introduce the genes needed for the production of a specific protein or improve the characteristics of a cell line.

The following methods are used to introduce foreign DNA into mammalian cells:

1. Electroporation
2. Lipofection
3. Microinjection
4. Fusion of mammalian cells with bacteria or viruses.

As the foreign DNA gets integrated into the mammalian cellular genome, the gene expresses to produce the desired protein. It is however, necessary to select the best producing recombinant cells by conventional methods using selectable marker genes.

The following selectable markers are used for choosing the transfected cells:

i. Viral thymidine kinase
ii. Bacterial dihydrofolate reductase
iii. Bacterial neomycin phosphotransferase

It has been possible to overproduce several proteins in mammalian cells through genetic manipulations e.g., tissue plasminogen activator, erythropoietin, interleukin-2, interferon-β, clotting factors VIII and IX, tumor necrosis factors. The recombinant mammalian cells are conveniently used for the production of monoclonal antibodies which have wide range of applications.