ANNAMALAI UNIVERSITY

FACULTY OF AGRICULTURE

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY

III. B. Sc (Ag.) **VI SEMESTER**

THEORY LECTURE NOTES

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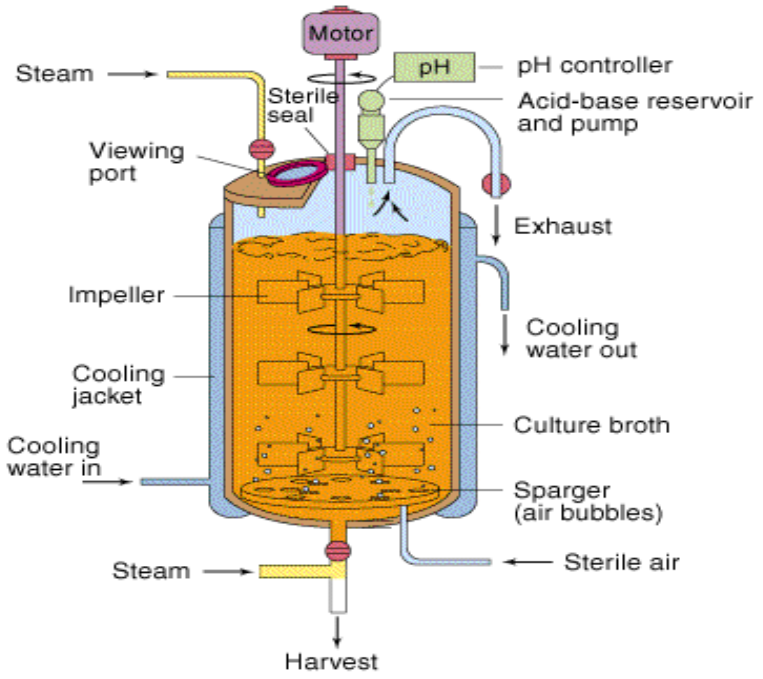
ADVANCED MICROBIAL BIOTECHNOLOGY (1 + 1)



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UNIT – I

Fermentation

Fermentation is a metabolic process that produces chemical changes in organic substrates through the action of enzymes. In biochemistry, it is narrowly defined as the extraction of energy from carbohydrates in the absence of respiration. In the context of food production, it may more broadly refer to any process in which the activity of microorganisms brings about a desirable change to a foodstuff or beverage.[1] The science of fermentation is known as zymology. In microorganisms, fermentation is the primary means of producing adenosine triphosphate (ATP) by the degradation of organic nutrients anaerobically. Humans have used fermentation to produce foodstuffs and beverages since the Neolithic age. For example, fermentation is used for preservation in a process that produces lactic acid found in such sour foods as pickled cucumbers, kombucha, kimchi, and yogurt, as well as for producing alcoholic beverages such as wine and beer. Fermentation also occurs within the gastrointestinal tracts of all animals, including humans. Below are some definitions of fermentation. They range from informal, general usages to more scientific definitions.

1. Preservation methods for food via microorganisms (general use).
2. Any process that produces alcoholic beverages or acidic dairy products (general use).
3. Any large-scale microbial process occurring with or without air (common definition used in industry).
4. Any energy-releasing metabolic process that takes place only under anaerobic conditions (becoming more scientific).
5. Any metabolic process that releases energy from a sugar or other organic molecule, does not require oxygen or an electron transport system, and uses an organic molecule as the final electron acceptor (most scientific).

Along with photosynthesis and aerobic respiration, fermentation is a way of extracting energy from molecules, but it is the only one common to all bacteria and eukaryotes. It is therefore considered the oldest metabolic pathway, suitable for an environment that does not yet have oxygen.

Yeast, a form of fungus, occurs in almost any environment capable of supporting microbes, from the skins of fruits to the guts of insects and mammals and the deep ocean, and they harvest sugar-rich materials to produce ethanol and carbon dioxide.

The basic mechanism for fermentation remains present in all cells of higher organisms. Mammalian muscle carries out the fermentation that occurs during periods of intense exercise where oxygen supply becomes limited, resulting in the creation of lactic acid. In invertebrates, fermentation also produces succinate and alanine.

Fermentative bacteria play an essential role in the production of methane in habitats ranging from the rumens of cattle to sewage digesters and freshwater sediments. They produce hydrogen, carbon dioxide, formate and acetate and carboxylic acids; and then consortia of microbes convert the carbon dioxide and acetate to methane. Acetogenic bacteria oxidize the acids, obtaining more acetate and either hydrogen or formate. Finally, methanogens (which are in the domain *Archea*) convert acetate to methane.[

Biochemistry of fermentation

Fermentation reacts NADH with an endogenous, organic electron acceptor. Usually this is pyruvate formed from sugar through glycolysis. The reaction produces NAD+ and an organic product, typical examples being ethanol, lactic acid, carbon dioxide, and hydrogen gas (H_2). However, more exotic compounds can be produced by fermentation, such as butyric acid and acetone. Fermentation products contain chemical energy (they are not fully oxidized), but are considered waste products, since they cannot be metabolized further without the use of oxygen.

Fermentation normally occurs in an anaerobic environment. In the presence of O_2, NADH, and pyruvate are used to generate ATP in respiration. This is called oxidative phosphorylation, and it generates much more ATP than glycolysis alone. For that reason, fermentation is rarely utilized when oxygen is available. However, even in the presence of abundant oxygen, some strains of yeast such as *Saccharomyces cerevisiae* prefer fermentation to aerobic respiration as long as there is an adequate supply of sugars (a phenomenon known as the Crabtree effect). Some fermentation processes involve obligate anaerobes, which cannot tolerate oxygen.

Although yeast carries out the fermentation in the production of ethanol in beers, wines, and other alcoholic drinks, this is not the only possible agent: bacteria carry out the fermentation in the production of xanthan gum.

Modes of operation

Most industrial fermentation uses batch or fed-batch procedures, although continuous fermentation can be more economical if various challenges, particularly the difficulty of maintaining sterility, can be met.

Batch fermentation

In a batch process, all the ingredients are combined and the reactions proceed without any further input. Batch fermentation has been used for millennia to make bread and alcoholic beverages, and it is still a common method, especially when the process is not well understood. However, it can be expensive because the fermentor must be sterilized using high pressure steam between batches. Strictly speaking, there is often addition of small quantities of chemicals to control the pH or suppress foaming.

Batch fermentation goes through a series of phases. There is a lag phase in which cells adjust to their environment; then a phase in which exponential growth occurs. Once many of the nutrients have been consumed, the growth slows and becomes non-exponential, but production of *secondary metabolites* (including commercially important antibiotics and enzymes) accelerates. This continues through a stationary phase after most of the nutrients have been consumed, and then the cells die.

Fed-batch fermentation

Fed-batch fermentation is a variation of batch fermentation where some of the ingredients are added during the fermentation. This allows greater control over the stages of the process. In particular, production of secondary metabolites can be increased by adding a limited quantity of nutrients during the non-exponential growth phase. Fed-batch operations are often sandwiched between batch operations.

Open fermentation

The high cost of sterilizing the fermentor between batches can be avoided using various open fermentation approaches that are able to resist contamination. One is to use a naturally evolved mixed culture. This is particularly favored in wastewater treatment, since mixed populations can adapt to a wide variety of wastes.

Thermophilic bacteria can produce lactic acid at temperatures of around 50 degrees Celsius, sufficient to discourage microbial contamination; and ethanol has been produced at a temperature of 70 °C. This is just below its boiling point (78 °C), making it easy to extract. Halophilic bacteria can produce bioplastics in hypersaline conditions. Solid-state fermentation adds a small amount of water to a solid substrate; it is widely used in the food industry to produce flavors, enzymes and organic acids.

Continuous fermentation

In continuous fermentation, substrates are added and final products removed continuously. There are three varieties: chemostats, which hold nutrient levels constant; turbidostats, which keep cell mass constant; and plug flow reactors in which the culture medium flows steadily through a tube while the cells are recycled from the outlet to the inlet. If the process works well, there is a steady flow of feed and effluent and the costs of repeatedly setting up a batch are avoided. Also, it can prolong the exponential growth phase and avoid byproducts that inhibit the reactions by continuously removing them. However, it is difficult to maintain a steady state and avoid contamination, and the design tends to be complex. Typically the fermentor must run for over 500 hours to be more economical than batch processors.

History of the use of fermentation

The use of fermentation, particularly for beverages, has existed since the Neolithic and has been documented dating from 7000–6600 BCE in Jiahu, China, 5000 BCE in India, Ayurveda mentions many Medicated Wines, 6000 BCE in Georgia, 3150 BCE in ancient Egypt, 3000 BCE in Babylon, 2000 BCE in pre-Hispanic Mexico, and 1500 BC in Sudan. Fermented foods have a religious significance in Judaism and Christianity. The Baltic god Rugutis was worshiped as the agent of fermentation.

Louis Pasteur in his laboratory

In 1837, Charles Cagniard de la Tour, Theodor Schwann and Friedrich Traugott Kützing independently published papers concluding, as a result of microscopic investigations, that yeast is a living organism that reproduces by budding. Schwann boiled grape juice to kill the yeast and found that no fermentation would occur until new yeast was added. However, a lot of chemists, including Antoine Lavoisier, continued to view fermentation as a simple chemical reaction and rejected the notion that living organisms could be involved.

This was seen as a reversion to vitalism and was lampooned in an anonymous publication by Justus von Liebig and Friedrich Wöhler.

The turning point came when Louis Pasteur (1822–1895), during the 1850s and 1860s, repeated Schwann's experiments and showed that fermentation is initiated by living organisms in a series of investigations. In 1857, Pasteur showed that lactic acid fermentation is caused by living organisms. In 1860, he demonstrated that bacteria cause souring in milk, a process formerly thought to be merely a chemical change, and his work in identifying the role of microorganisms in food spoilage led to the process of pasteurization. In 1877, working to improve the French brewing industry, Pasteur published his famous paper on fermentation, "*Etudes sur la Bière*", which was translated into English in 1879 as "Studies on fermentation". He defined fermentation (incorrectly) as "Life without air", but correctly showed that specific types of microorganisms cause specific types of fermentations and specific end-products.

Although showing fermentation to be the result of the action of living microorganisms was a breakthrough, it did not explain the basic nature of the fermentation process, or prove that it is caused by the microorganisms that appear to be always present. Many scientists, including Pasteur, had unsuccessfully attempted to extract the fermentation enzyme from yeast. Success came in 1897 when the German chemist Eduard Buechner ground up yeast, extracted a juice from them, then found to his amazement that this "dead" liquid would ferment a sugar solution, forming carbon dioxide and alcohol much like living yeasts.[41] Buechner's results are considered to mark the birth of biochemistry.

The "unorganized ferments" behaved just like the organized ones. From that time on, the term enzyme came to be applied to all ferments. It was then understood that fermentation is caused by enzymes that are produced by microorganisms. In 1907, Buechner won the Nobel Prize in chemistry for his work .Advances in microbiology and fermentation technology have continued steadily up until the present. For example, in the 1930s, it was discovered that microorganisms could be mutated with physical and chemical treatments to be higher-yielding, faster-growing, tolerant of less oxygen, and able to use a more concentrated medium. Strain selection and hybridization developed as well, affecting most modern food fermentations.

Industrial utilization of microorganisms

I. Fermentation technology:

Industrial fermentation is the intentional use of fermentation by microorganisms such as bacteria and fungi as well as eukaryotic cells like CHO cells and insect cells, to make products useful to humans.

Fermented products have applications as food as well as in general industry. Some commodity chemicals, such as acetic acid, citric acid, and ethanol are made by fermentation. The rate of fermentation depends on the concentration of microorganisms, cells, cellular components, and enzymes as well as temperature, pH and for aerobic fermentation oxygen. Product recovery frequently involves the concentration of the dilute solution. Nearly all commercially produced enzymes, such as lipase, invertase and rennet, are made by fermentation with genetically modified microbes. In some cases, production of biomass itself is the objective, as in the case of baker's yeast and lactic acid bacteria starter cultures for cheesemaking. In general, fermentations can be divided into four types:

- Production of biomass (viable cellular material)
- Production of extracellular metabolites (chemical compounds)
- Production of intracellular components (enzymes and other proteins)
- Transformation of substrate (in which the transformed substrate is itself the product)

These types are not necessarily disjoint from each other, but provide a framework for understanding the differences in approach. The organisms used may be bacteria, yeasts, molds, algae, animal cells, or plant cells. Special considerations are required for the specific organisms used in the fermentation, such as the dissolved oxygen level, nutrient levels, and temperature.

In most industrial fermentations, the organisms or eukaryotic cells are submerged in a liquid medium; in others, such as the fermentation of cocoa beans, coffee cherries, and miso, fermentation takes place on the moist surface of the medium. There are also industrial considerations related to the fermentation process. For instance, to avoid biological process contamination, the fermentation medium, air, and equipment are sterilized. Foam control can be achieved by either mechanical foam destruction or chemical anti-foaming agents.

Several other factors must be measured and controlled such as pressure, temperature, agitator shaft power, and viscosity. An important element for industrial fermentations is scale up. This is the conversion of a laboratory procedure to an industrial process. It is well established in the field of industrial microbiology that what works well at the laboratory scale may work poorly or not at all when first attempted at large scale. It is generally not possible to take fermentation conditions that have worked in the laboratory and blindly apply them to industrial-scale equipment.

Although many parameters have been tested for use as scale up criteria, there is no general formula because of the variation in fermentation processes. The most important methods are the maintenance of constant power consumption per unit of broth and the maintenance of constant volumetric transfer rate.

Phases of growth

Fermentation begins once the growth medium is inoculated with the organism of interest. Growth of the inoculum does not occur immediately. This is the period of adaptation, called the lag phase. Following the lag phase, the rate of growth of the organism steadily increases, for a certain period—this period is the log or exponential phase .After a phase of exponential growth, the rate of growth slows down, due to the continuously falling concentrations of nutrients and/or a continuously increasing (accumulating) concentrations of toxic substances. This phase, where the increase of the rate of growth is checked, is the deceleration phase. After the deceleration phase, growth ceases and the culture enters a stationary phase or a steady state. The biomass remains constant, except when certain accumulated chemicals in the culture lyse the cells (chemolysis). Unless other micro-organisms contaminate the culture, the chemical constitution remains unchanged. If all of the nutrients in the medium are consumed, or if the concentration of toxins is too great, the cells may become senescent and begin to die off. The total amount of biomass may not decrease, but the number of viable organisms will decrease.

Fermentation medium

The microbes or eukaryotic cells used for fermentation grow in (or on) specially designed growth medium which supplies the nutrients required by the organisms or cells. A variety of media exist, but invariably contain a carbon source, a nitrogen source, water, salts, and micronutrients.

In the production of wine, the medium is grape must. In the production of bio-ethanol, the medium may consist mostly of whatever inexpensive carbon source is available.

Carbon sources are typically sugars or other carbohydrates, although in the case of substrate transformations (such as the production of vinegar) the carbon source may be an alcohol or something else altogether. For large scale fermentations, such as those used for the production of ethanol, inexpensive sources of carbohydrates, such as molasses, corn steep liquor,[8] sugar cane juice, or sugar beet juice are used to minimize costs. More sensitive fermentations may instead use purified glucose, sucrose, glycerol or other sugars, which reduces variation and helps ensure the purity of the final product. Organisms meant to produce enzymes such as beta galactosidase, invertase or other amylases may be fed starch to select for organisms that express the enzymes in large quantity.

Fixed nitrogen sources are required for most organisms to synthesize proteins, nucleic acids and other cellular components. Depending on the enzyme capabilities of the organism, nitrogen may be provided as bulk protein, such as soy meal; as pre-digested polypeptides, such as peptone or tryptone; or as ammonia or nitrate salts. Cost is also an important factor in the choice of a nitrogen source. Phosphorus is needed for production of phospholipids in cellular membranes and for the production of nucleic acids. The amount of phosphate which must be added depends upon the composition of the broth and the needs of the organism, as well as the objective of the fermentation. For instance, some cultures will not produce secondary metabolites in the presence of phosphate.

Growth factors and trace nutrients are included in the fermentation broth for organisms incapable of producing all of the vitamins they require. Yeast extract is a common source of micronutrients and vitamins for fermentation media. Inorganic nutrients, including trace elements such as iron, zinc, copper, manganese, molybdenum and cobalt are typically present in unrefined carbon and nitrogen sources, but may have to be added when purified carbon and nitrogen sources are used. Fermentations which produce large amounts of gas (or which require the addition of gas) will tend to form a layer of foam, since fermentation broth typically contains a variety of foam-reinforcing proteins, peptides or starches.

To prevent this foam from occurring or accumulating, antifoaming agents may be added. Mineral buffering salts, such as carbonates and phosphates, may be used to stabilize pH near optimum. When metal ions are present in high concentrations, use of a chelating agent may be necessary.

Developing an optimal medium for fermentation is a key concept to efficient optimization. One-factor-at-a-time (OFAT) is the preferential choice that researchers use for designing a medium composition. This method involves changing only one factor at a time while keeping the other concentrations constant. This method can be separated into some sub groups. One is Removal Experiments. In this experiment all the components of the medium are removed one at a time and their effects on the medium are observed.

Supplementation experiments involve evaluating the effects of nitrogen and carbon supplements on production. The final experiment is a replacement experiment. This involves replacing the nitrogen and carbon sources that show an enhancement effect on the intended production. Overall OFAT is a major advantage over other optimization methods because of its simplicity.

Production of biomass

Microbial cells or biomass is sometimes the intended product of fermentation. Examples include single cell protein, baker's yeast, lactobacillus, *E. coli*, and others. In the case of single-cell protein, algae is grown in large open ponds which allow photosynthesis to occur. If the biomass is to be used for inoculation of other fermentations, care must be taken to prevent mutations from occurring.

Production of extracellular metabolites

Metabolites can be divided into two groups: those produced during the growth phase of the organism, called **primary metabolites** and those produced during the stationary phase, called **secondary metabolites**. Some examples of primary metabolites are ethanol, citric acid, glutamic acid, lysine, vitamins and polysaccharides. Some examples of secondary metabolites are penicillin, cyclosporin A, gibberellin, and lovastatin.

Primary metabolites

Primary metabolites are compounds made during the ordinary metabolism of the organism during the growth phase. A common example is ethanol or lactic acid, produced during glycolysis.

Citric acid is produced by some strains of *Aspergillus niger* as part of the citric acid cycle to acidify their environment and prevent competitors from taking over. Glutamate is produced by some *Micrococcus* species, and some *Corynebacterium* species produce lysine, threonine, tryptophan and other amino acids. All of these compounds are produced during the normal "business" of the cell and released into the environment. There is therefore no need to rupture the cells for product recovery.

Secondary metabolites

Secondary metabolites are compounds made in the stationary phase; penicillin, for instance, prevents the growth of bacteria which could compete with *Penicillium* molds for resources. Some bacteria, such as *Lactobacillus* species, are able to produce bacteriocins which prevent the growth of bacterial competitors as well. These compounds are of obvious value to humans wishing to prevent the growth of bacteria, either as antibiotics or as antiseptics (such as gramicidin S). Fungicides, such as griseofulvin are also produced as secondary metabolites.

Typically secondary metabolites are not produced in the presence of glucose or other carbon sources which would encourage growth, and like primary metabolites are released into the surrounding medium without rupture of the cell membrane. In the early days of the biotechnology industry, most biopharmaceutical products were made in *E. coli*; by 2004 more biopharmaceuticals were manufactured in eukaryotic cells, like CHO cells, than in microbes, but used similar bioreactor systems. Insect cell culture systems came into use in the 2000s as well.

Production of intracellular components

Of primary interest among the intracellular components are microbial enzymes: catalase, amylase, protease, pectinase, cellulase, hemicellulase, lipase, lactase, streptokinase and many others. Recombinant proteins, such as insulin, hepatitis B vaccine, interferon, granulocyte colony-stimulating factor, streptokinase and others are also made this way. The largest difference between this process and the others is that the cells must be ruptured (lysed) at the end of fermentation, and the environment must be manipulated to maximize the amount of the product. Furthermore, the product (typically a protein) must be separated from all of the other cellular proteins in the lysate to be purified.

Transformation of substrate

Substrate transformation involves the transformation of a specific compound into another, such as in the case of phenylacetylcarbinol, and steroid biotransformation, or the transformation of a raw material into a finished product, in the case of food fermentations and sewage treatment.

Food fermentation

Ancient fermented food processes, such as making bread, wine, cheese, curds, idli, dosa, etc., can be dated to more than seven thousand years ago. They were developed long before man had any knowledge of the existence of the microorganisms involved. Some foods such as Marmite are the byproduct of the fermentation process, in this case in the production of beer.

Ethanol fuel

Fermentation is the main source of ethanol in the production of ethanol fuel. Common crops such as sugar cane, potato, cassava and corn are fermented by yeast to produce ethanol which is further processed to become fuel.

Sewage treatment

In the process of sewage treatment, sewage is digested by enzymes secreted by bacteria. Solid organic matters are broken down into harmless, soluble substances and carbon dioxide. Liquids that result are disinfected to remove pathogens before being discharged into rivers or the sea or can be used as liquid fertilizers. Digested solids, known also as sludge, is dried and used as fertilizer. Gaseous byproducts such as methane can be utilized as biogas to fuel electrical generators. One advantage of bacterial digestion is that it reduces the bulk and odor of sewage, thus reducing space needed for dumping. The main disadvantage of bacterial digestion in sewage disposal is that it is a very slow process.

Agricultural feed

A wide variety of agro-industrial waste products can be fermented to use as food for animals, especially ruminants. Fungi have been employed to break down cellulosic wastes to increase protein content and improve *in vitro* digestibility.

Industrial fermentation process:

The basic requirements of any industrial fermentation process include microorganisms, the medium (substrate), the production and recovery (Equipments & Process).

a) The microorganisms:

A suitable microorganism is a critical requisite for any fermentation process. The most suitable organism is sought by screening (identifying) from a population or creating specific strains of microorganisms that will yield high quantities of the desired product by genetic engineering. The strains thus selected should have relatively stable characteristics and the ability to grow rapidly and vigorously. The strain should essentially be nonpathogenic and a non-producer of any unwanted by products or toxins.

b) The medium

The design of optimal production process includes defining the substrate mixture containing least expensive compounds that are readily available and produce the highest yield of the desired product. In several instances it has been found practicable to utilize nutrient containing wastes from the dairy industry (whey), the paper industry (waste liquors) and other commercial operations.

Culture Media:. Physical Type of Culture Media:

Liquid, semisolid and solid media are routinely used for growth of micro-organisms.

(i) Liquid Media:

It is also called as broth and contains only dissolved nutrients in water. Liquid media are used for growth of pure batch cultures for fermentation studies and various other purposes while solidified media can be used widely for isolation of pure cultures for estimating viable microbial population and a variety of other purposes. The usual gelling agent for solid or semisolid medium is agar, a hydro colloid derived from red algae. Agar is used because of its unique physical properties. One is that it melts at about 96°C and remains liquid until cooled to 40 to 45°C. Thus after being melted in boiling water, it can be cooled to a temperature that is tolerated by human hands as well as microbes. Finally agar is excellent hardening agent because most microorganisms cannot metabolize it. It can be aided in light medial at consequence 1.5 to 2.0 permuted. Silca gel sometimes can also be substituted for agar as solidifying agent. Silca gel is made from silic acid and gel formed from this acid is completely resistant to microbial breakdown.

Culture Media: Type # 2. Chemical Type of Culture Media:

A medium in which the exact chemical composition is known is called chemically defined (synthetic) medium. It can be in liquid form (broth) or solidified by agents such as agar. Chemically defined media are often used for cultivation of autotrophs and are also useful. Simple peptone water, medium, 1% peptone with 0.5% NaCI in water may be considered semisynthetic medium since its composition is approximately known. For defining the nutritional requirement of heterotrophs. Typically they contain simple sugar as the carbon and energy source, an inorganic Nitrogen source, various mineral salts and if necessary growth factors (purified amino acids vitamins, purine and pyrimidines).

A media in which exact chemical composition are not known is called complex (non-synthetic medium). It usually contains complex material of biological origin etc. such as blood or milk or peptone yeast extract and beef extract. Complex media provide full range of growth factor that may be required by an organism so they may be used to cultivate unknown microorganisms or whose nutritional requirement is complex (i.e., organism that requires lot of growth factor). This is the situation with fastidious organisms that have complex nutrition requirement; they may even require a medium containing blood or serum. Potato extract agar, Sail extract agar, Oatmeal agar, nutrient broth and tryptic soya broth are commonly used complex media after cultivation of heterothophic microorganism.

Media Used For the Growth of Microorganisms

The media used for the growth of microorganisms in industrial fermentation must contain all the elements in a suitable form for the synthesis of cellular substances as well as the metabolic products. While designing a medium, several factors must be taken into consideration. The most important among them is the ultimate product desired in the fermentation. For growth-linked products (primary metabolites e.g. ethanol, citric acid), the product formations is directly dependent on the growth of the organisms, hence the medium should be such that it supports good growth. On the other hand, for products which are not directly linked to the growth (secondary metabolites e.g. antibiotics, alkaloids, gibberellins), the substrate requirements for product formation must also be considered. In the laboratory, pure defined chemicals may be used for culturing microorganisms.

However, for industrial fermentations, undefined and complex substrates are frequently used for economic reasons. Cheaper substrates are advantageous since they minimize the production cost of the fermented products. Wastes from agriculture, and byproducts of other industries are generally preferred, although they are highly variable in composition. Raw materials used in fermentation largely depend on their cost at a particular time, since there are seasonal variations. The choice of the medium is very critical for successful product formation. For industrial fermentation, the microorganisms, in general, utilize a luxury metabolism. Therefore, good production yields are expected with an abundant supply of carbon and nitrogen sources, besides requisite growth factors. The media used in fermentation processes may be synthetic or crude.

Synthetic media: Media with all the requisite constituents in a pure form in the desired proportion represents synthetic media. Use of this type of media in fermentations is not practicable.

Crude media: The non-synthetic media with naturally available sources are better suited for fermentation. In practice, crude media with an addition of requisite synthetic constituents is ideal for good product yield in fermentation. The most frequently used substrates for industrial fermentation with special reference to the supply of carbon and nitrogen sources and growth factors are briefly described below.

Substrates Used As Carbon Sources: Carbohydrates constitute the most predominant source of energy in fermentation industry. Refined and pure carbohydrates such as glucose or sucrose are rarely used for economic reasons.

Molasses: Molasses is a byproduct of sugar industry and is one of the cheapest sources of carbohydrates. Sugar cane molasses (sucrose around 48%) and sugar beet molasses (sucrose around 33%) are commonly used. Besides being rich in sugar, molasses also contain nitrogenous substances, vitamins and trace elements. There occurs variation in the composition of the molasses which mostly depends on the climatic conditions and production process. Hydrol molasses, a byproduct in glucose production from corn, is also used as a fermentation substrate.

Malt extract: Malt extract, an aqueous extract of malted barley, contains about 80% carbohydrates (glucose, fructose, sucrose, and maltose). Nitrogen compounds constitute around 4.5% (proteins, peptides, amino acids, purines, pyrimidine's).

Starch, dextrin and cellulose: The polysaccharides-starch, dextrin and cellulose can be metabolised by microorganisms. They are frequently used for the industrial production of alcohol. Due to its wide availability and low cost, the use of cellulose for alcohol production is extensively studied.

Whey: Whey is a byproduct of dairy industry and is produced worldwide. Most of it is consumed by- humans and animals. Whey is a reasonably good source of carbon for the production of alcohol, single-cell protein, vitamin B_{12}, lactic acid and gibberellic acid. Storage of whey is a limiting factor for its widespread use in fermentation industry.

Methanol and ethanol: Some of the microorganisms are capable of utilizing methanol and/or ethanol as carbon source. Methanol is the cheapest substrate for fermentation. However, it can be utilized by only a few bacteria and yeasts. Methanol is commonly used for the production of single-cell protein. Ethanol is rather expensive. However, at present it is used for the production of acetic acid.

Substrates Used As Nitrogen Sources:

The nitrogen supply to the fermentation microorganisms may come from inorganic or organic sources. **Inorganic nitrogen sources:**

Ammonium salts and free ammonia are cheap inorganic nitrogen sources, particularly in industrialised countries. However, not all the microorganisms are capable of utilizing them, hence their use is limited.

Organic nitrogen sources: Urea is fairly a good source of nitrogen. However, other cheaper organic forms of nitrogen sources are preferred.

Corn steep liquor: This is formed during starch production from corn. Corn steep liquor is rich in nitrogen (about 4%) and is very efficiently utilized by microorganisms. It is rich in several amino acids (alanine, valine, methionine, arginine, threonine, glutamate).

Yeast extracts: They contain about 8% nitrogen and are rich in amino acids, peptides and vitamins. Glucose formed from glycogen and trehalose during yeast extraction is a good carbon source. Yeast extracts are produced from baker's yeast through autolysis (at 50-55°C) or through plasmolysis (high concentration of NaCI). Yeast extracts are very good sources for many industrially important microorganisms.

Soy meal: After extracting the soy bean oil from the soy bean seeds, the left out residue is soy meal. It is rich in proteins (about 50%) as well as carbohydrates (about 30%) contents. Soy meal is often used in antibiotic production.

Peptones: The protein hydro-lysates are collectively referred to as peptones, and they are good sources for many microorganisms. The sources of peptones include meat, soy meal, peanut seeds, cotton seeds and sunflower seeds.
The proteins namely casein, gelatin and keratin can also be hydrolysed to yield peptones. In general, peptones derived from animal sources have more nitrogen content while those from plant sources have more carbohydrate content. Peptones are relatively more expensive, hence not widely used in industries.

Sources of Growth Factors:

Some of the microorganisms are not capable of synthesizing one or more growth factors such as vitamins. These growth factors are very expensive in pure form, hence crude sources are preferred. Yeast extract is a rich source of almost all growth factors. Generally, the substrates derived from plant or animal sources in a crude form are reasonably rich in mineral content. Sometimes, however mineral (phosphate, sulfate) supplementation may be required.

Type of Culture Media:

No single medium or set of conditions can support the growth of all the different types of organisms that occur in nature. To cultivate, recognize, enumerate and isolate certain types of microorganism many special purpose media are needed.

On the basis of their application and functions these media are classified into following types:

Selective media provide nutrients that enhance growth and predominance of particular type of microorganism and suppresses all other microorganisms that are present in culture. This medium is best used for isolating specific organism from a mixed natural population. For instance, cellulose utilizing micro-organism alone will grow in medium containing only cellulose as a carbon and energy source. Endo agar, eosin methylene blue agar and Mac Conkey agars are three media widely used for detection of E. coli and related bacteria in water supply. These media contain dyes that inhibit the growth of gram +ve bacteria but allow gram negative bacteria to grow. Physical condition such as pH and temperature also used to render its selectivity for growth of microorganism in media. As an example, gonorrhea causing Nosseria gononhoeae, can be grown and isolated from medium containing certain antibiotics. Thus antibiotics exhibit growth of contaminating bacteria.

(i) Differential Media:

Differential media is used for the differentiation of various kinds of microorganisms on the basis of appearance on the medium and even permit tentative identification of microorganism. These media allow certain microorganisms to produce macroscopically distinct colonies or characteristic zone around colonies, which are differentiating these organisms from others in the samples. This medium contains certain indicator or reagent or supplement which may allow such differentiation. For example, if a mixture of bacteria is inoculated on to a blood-containing agar mediums (blood agar), some of bacteria may hemolyze (destroy) the red blood cells, others do not. Thus we can distinguish between hemolytic and non-hemolytic bacteria on the same medium. Mac Conkey's agar, contains lactose and a dye which turns when pH drops below. Any microorganism that can ferment lactose produces an acid end product that Lowers the pH and causes colony to turn red. Microorganism that fails to ferment lactose produces colour-less colonies. Dye in Mac Conkye's agar also inhibits the growth of gram +ve bacteria. This medium is, therefore, both selective and differential.

(ii) Enrichment Medium:

Enrichment medium is that in which nutritional environment is adjusted in such a manner as to enhance selectively the growth of certain bacterial type with in a gives mixed inoculum. For instance addition of extract of plant and animal tissue to nutrient broth and nutrient agar media provides additional nutrient and media starts favouring the growth of fasticious herotropic bacteria. In the case of delicate micro-organisms like goner which may not survive the time taken for transporting the specimen to the laboratory or may be overgrown by non-pathogens (such as dysentery or cholera organism in feces), special media are devised for transporation of the specimens. These are termed transport media for example, Stuart's medium a non-nutrient soft agar gel containing a reducing agent to prevent oxidation, and charcoal to neutralize acetone bacterial inhibitors for gonococci and buffer acid glycol saline for enteric bacilli.

c) The product and recovery:

Fermenters with huge capacities should be designed with optimal environmental conditions to produce maximal yields of the product. The final product will be mixed numerous other chemicals and live and dead microbial cells. The process of recovery of the final product in the pure form is the final important step in industrial fermentation.

Some important microbial species used in industry

Classes of final product	Microorganism	Actual final product
Alcohols and Solvents	*Saccharomyces cerevisiae* *Kluveromyces fragilis.* *Clostridium acetobutylicum*	Ethanol Ethanol Acetone and Butanol
Organic Acids	*Aspergillus niger* *Lactobacillus delbrueckii.*	Citric Acid Lactic Acid
Enzymes	*Aspergillus niger* *Bacillus subtilis* *Trichoderma reesi* *Saccharomyces cerevisiae* *S. lipolytica* *Aspergillus* spp. *Bacillus* spp.	Glucoamylase Amylase Cellulose Invertase Lipase Pectinases Proteases
Amino acids	*Cornybacterium glutamicum* *Brevibacterium* spp.	L-lysine Glutamic acid
Vitamins	*Ashbya gossypii* *Pseudomonas denitrificans* *Proionibacterium shermanii*	Riboflavin Vitamin B_{12} Vitamin B_{12}
Polysaccharides	*Leuconostoc mesenteroides* *Xanthomonas campestris*	Dextran Xanthan

Bioinsecticides	*Bacillus thuringiensis* *Bacillus popilliae*	Anti-larval products for control of mosquitoes etc.
Food supplements	*Methane oxidizing* bacteria	Single cell protein (SCP)
Pharmaceuticals	*Penicillium chrysogenum* *Cephalosporium acremonium* *Streptomyces* spp. *Bacillus brevis* *B. subtilis* *B. polymyxa* *Rhizopus nigricans* *Escherchia coli* (by recombinant DNA technology)	Penicillins Cephalosporins Amphoterecin B, Kanamycins, tetracyclines, etc., Geramicidin-S Bacitracin Polymyxin-B Steroids (by steroid transformation) Insulin, Human growth hormone, Somatostatin, Interferon.

The Fermentation Processes:

Industrial fermentation processes may be divided into two main types: 1. Batch Fermentation and 2. Continuous Fermentation.

1. Batch Fermentation;

It is a discontinuous process and the fermentor has to be cleaned after each process and a fresh batch is started. The fermentor tank if filled with raw material, the temperature and pH is suitably adjusted and the microbial inoculum is introduced to start the fermentation process. Proper sterility has to be maintained in the fermentor. After a fixed time the fermentation process is stopped and the product is harvested. A next batch of fermentation follows. In a batch fermentor, the growth of the microorganisms follows the characteristic growth curve with a lag phase followed by a log phase finally reaching the stationary phase due to limitation of nutrient and other factors. This is the time to stop fermentation and harvest the product.

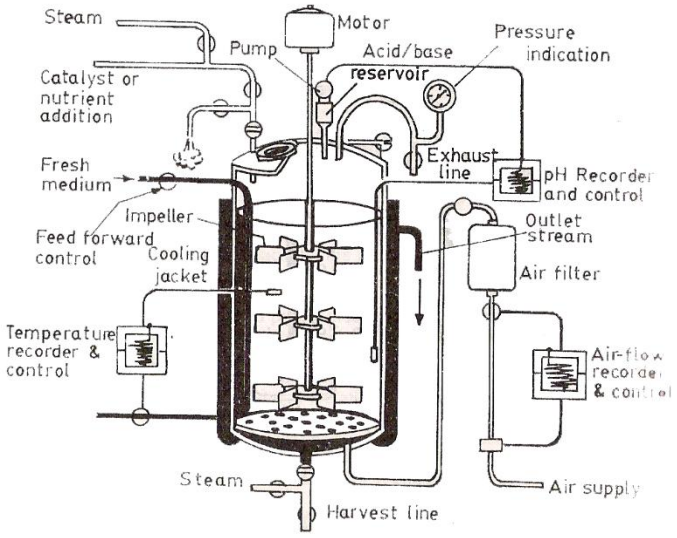


Fig. 16.2: A flow-through fermentor or continuous fermentor.

2. Continuous Fermenters:

It is a continuous process where the nutrient is continuously added to the fermentor at a fixed rate. The organisms are continuously maintained in the logarithmic phase. The products are recovered continuously, and this is also called as flow-through fermentors. This process is more prone for contamination, making difficult to maintain sterility. Nevertheless, this is the more popular industrial process.

Aerobic Fermentors:

The industrial fermentors need to be properly aerated with sterile air. Most industrial fermentations are aerobic processes and not anaerobic as mentioned earlier. The organisms grown in submerged cultures in modern fermentors are aerated by forcing sterilized air.

Anaerobic Fermentors:

Anaerobic fermentors are designed almost the same way as the aerobic fermentors but do not have the continuous air supply. They may require an initial agitation with air to mix the nutrients and a final agitation to properly distribute the products, but they do not require continuous agitation with sterile air.

Recovery of Product:

- Distillation
- Centrifugation
- Filtration
- Chromatographic separation
- Quality control.

Alcoholic Fermentation:

Conversion of sugar into alcohol by microbial enzymes is called alcoholic fermentation. The fermentation process carried out by yeasts can be represented by the following equation:

$$C_6H_{12}O_6 \xrightarrow[\text{enzymes}]{\text{Yeast}} 2C_2H_5OH+2CO_2$$

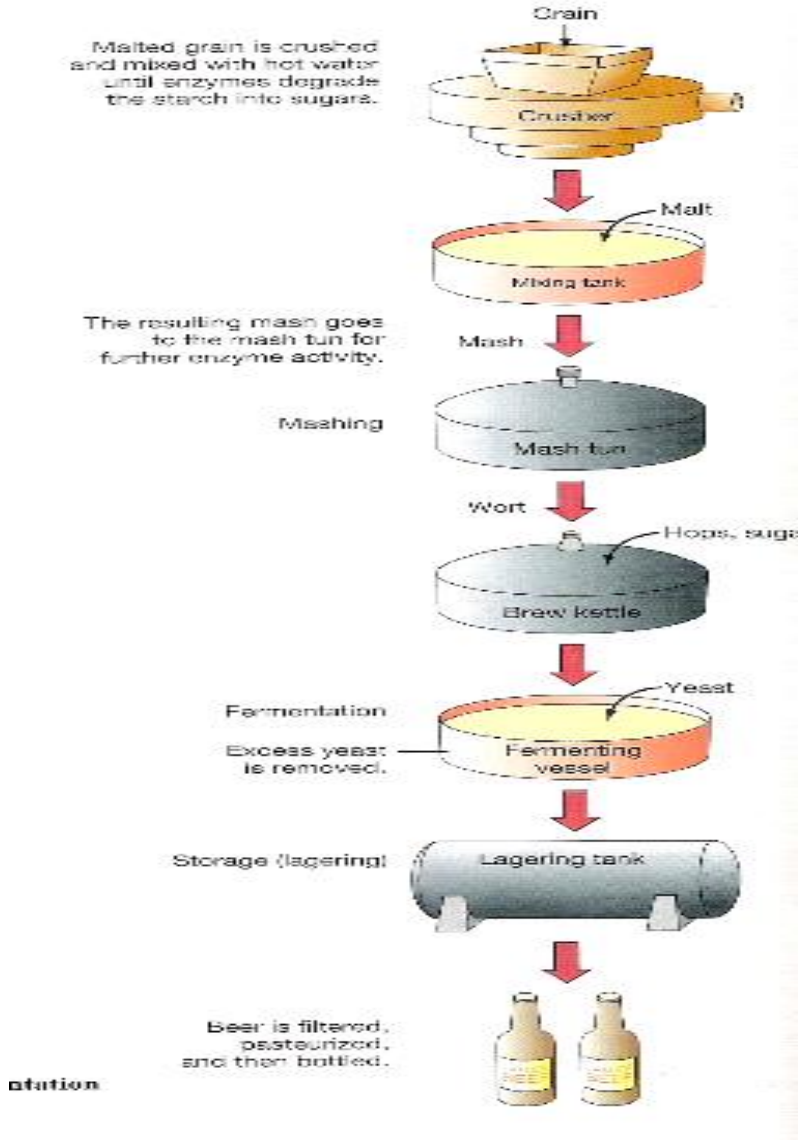
The process is anaerobic. Strains of *Saccharomyces cerevisiae* with capacity to produce high yields of alcohol are used. The industrial alcohol is produced from inexpensive substrates such as wastes of dairy and food industry. However, preparation of alcoholic beverages requires specific substrates as the flavor, colour and aroma of the beverage depends on the substrates. The alcoholic beverages also differ with respect to the production process, even though the microorganism involved is the same in all cases. Some important beverages are discussed here.

Beer:

Beer is so called because it is derived from fermentation of barely. The starting substrate is barley malt which is germinated barley grains that are dried and ground. Barley malt contains a mixture of amylases and proteinases. Production of beer entirely from malted barely is done in some European countries but in most places beer is made from malted barley to which are added corn, rice or wheat are added as adjuncts. These contain carbohydrates for ethanol production. During the **'mashing process'**, amylases present in malt hydrolyze starches and other polysaccharides to sugars. The mash is heated to a temperature of up to 70 ºC to allow rapid enzymatic conversion. The insoluble materials in the mash settle down leaving a clear liquid above called **'wort'**. The wort is cooked with **hops** (dried flowers of *Humulus lupulus*) to get the typical beer flavour.

During cooking, the enzymes and also the microorganisms get inactivated. The wort is then fermented with the yeast *Saccharomyces carlsbergensis*. The yeast cells flocculate and settle to the bottom as fermentation process is accompanied by extensive foaming due to production. The fermentation process is accompanied by extensive foaming due to production of carbon dioxide. The temperature is maintained at 6 to 12 ºC and the process may take 1 to 2 weeks. The product requires aging to get the required flavor and aroma. During the aging process the harsh flavor and other undesirable characteristics of the product get reduced. Aging takes 2 weeks to several months.

The final product is filtered and injected with carbon dioxide during bottling. The bottled or canned beers are pasteurized at 60-61 °C. The final alcohol content of beer ranges from 3.8 to 5 per cent.



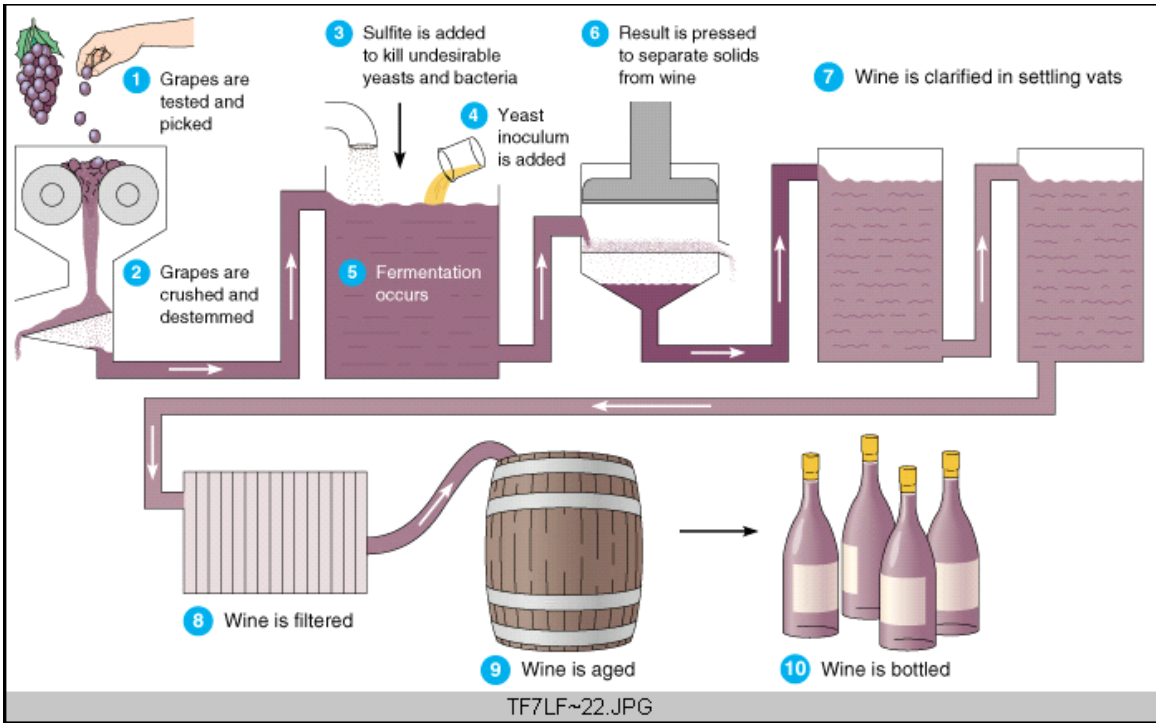
Wine making:

Wine is produced by the fermentation of grape juice. The organism *Saccharomyces cerevisiae var. ellipsoides* is naturally present on the surface of most fruits. In industrial wine production the natural or 'wild-type' yeasts should be inactivated by sulphur dioxide fumigation or other methods so that they will not compete with the defined yeast used for fermentation.

Red wines are made from red grapes and white wines from green grapes. The grapes are crushed to form juice or 'Grape must'. The 'must' is inoculated with a specific strain of *S. cerevisiae ellipsoides*. To start the fermentation process the 'must' is agitated with air to increase the proliferation of yeast cells. The fermentation is then allowed to continue anaerobically. The final alcohol concentration depends on the sugar content of the grapes and the alcohol tolerance of the yeast strains. Red wines are generally fermented at 24 to 27 ºC for 3 to 5 days. White wines take 7 to 14 days at 10-21 ºC. The colour of red wine is due to the pigments in the skin of the red grapes which are progressively extracted by the alcohol produced. The final yield of ethanol in wines varies from 7 to 15 per cent.

To achieve the particular flavor of wines, it is necessary to allow wines to age. Traditionally, wines are allowed to age in wood (oak) casks but in modern industry, they are bottled. During aging some fermentation of malic acid of grape juice is carried out by lactobacilli reducing acidity of the wine.

Wines are of different types. Sweet wines contain some unfermented sugar whereas dry wines contain little sugar. Fortified or dessert wines contain added alcohol, the total alcohol content reaching 19-21 per cent. Champagne and other sparkling wines contain carbon dioxide. During the fermentation process, the carbon dioxide produced is normally allowed to escape. In sparkling wines it is retained. In some varieties of champagnes, carbon dioxide is re injected into the final product to make it effervescent. In the French champagne, the wine is fermented in bottles and after the fermentation is complete, the bottles are inverted. The yeast cells settle at the neck of the bottles. The yeasts are then frozen and removed so that the carbon dioxide produced is retained.



Distilled Liquor:

Distilled liquor contains a higher percentage of alcohol than wine and beer. In the normal fermentation process a level of 18 per cent alcohol can be reached but not higher as a concentration higher than this can be detrimental to yeast cells themselves. The production of strong liquor therefore requires distillation. The initial fermentation process is similar to the production of beer beginning with the mashing process. Various substrates are used in the production of distilled liquor.

Malt whisky is produced by the fermentation of malted barley. Grain whisky is produced from a mixture of malted and un malted barley and corn. **Scotch whisky** is a blend of malt and grain whisky many other whiskeys are produced by continuous process. Bourbon or corn whisky uses corn mash. Irish whisky is obtained from rye mash.

Brandy is made from grapes. The yeast used is a different strain from that used for wine production, having greater ability to yield and tolerate alcohol.

Rum is produced by the fermentation of sugarcane molasses.

Gin is prepared by extracting berries of Junipers with alcohol and by further distillation. The juniper berries impart the particular flavor.

Antibiotics

Definition: Antibiotics are substances of microbial origin or secondary metabolites of certain microorganisms which at low concentrations either kill or inhibit the growth of other microorganisms.

Many microorganisms and plants excrete products that are not related to the basic metabolism of the producing organism; these products are called secondary metabolites. Bacteria and fungi produce a lot of secondary substances termed secondary metabolites. Many of these substances play important role as therapeutics, stimulants, feed additives etc. and because of which microorganisms gained industrial importance.

The discovery of penicillin and other antibiotics opened up a new area for industrial microbiology. There are no practical limitations to new discoveries, modifications and applications of secondary metabolites.

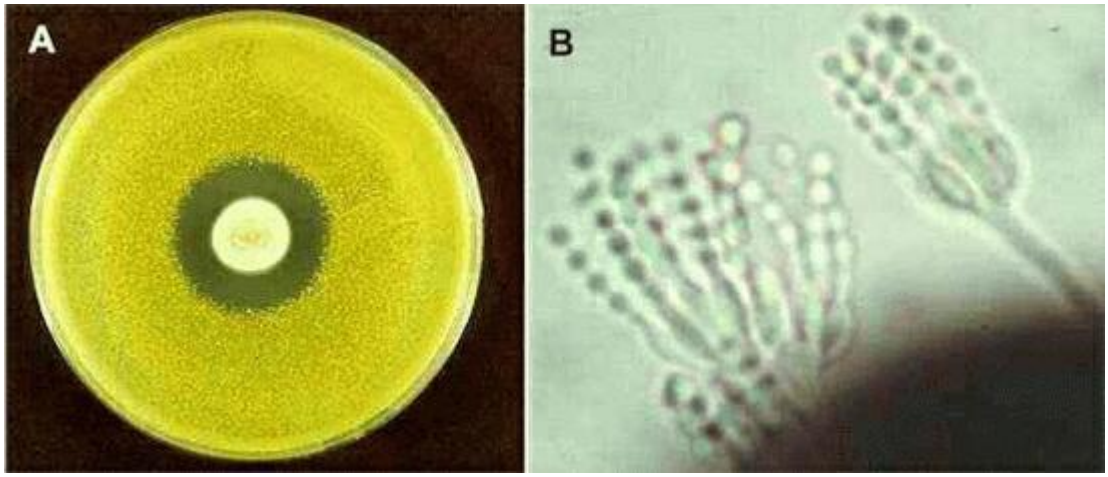
Production of antibiotics: The history of antibiotics originated with the observation of Alexander Fleming in 1928 that a fungal colony (*Penicillium notatum*) inhibited the growth of *Staphylococcus aureus* in a plate. It was observed that the compound excreted by the fungus diffused in the agar and inhibited the growth of the bacterium. Later the compound was named as penicillin. The ability of producing antibiotic substances has been found mainly in fungi (Aspergillales), actinomycetes and in few bacteria. In 1944 Selman A. Waksmann in Rutgers University discovered the antibiotic streptomycin produced by *Streptmyces sp.* The streptmycetes are remarkable for the chemical diversity of antibiotics that they produce.

Antibiotics are produced by special synthetic pathways, which are included in what is known as secondary metabolism. The significance of antibiotics for the producing organisms is almost completely unknown. It is hypothetically said that antibiotics must confer some advantages to their producers in their natural habitat, perhaps by favoring them in competition for limited substrates. Presently, antibiotics and other secondary metabolites, whose immediate usefulness to the producing organisms are not discernible. However such products show that the secondary metabolism of bacteria and fungi can be fruitful subjects for research aimed at understanding the evolution of microorganisms.

The first antibiotic was discovered more or less by chance because of the production of inhibition zones around the growth of the target organism.

Penicillin: It is produced by *Penicillium notatum* and *P.chrysogenum* and a few other fungi. The several kinds of penicillin synthesized by various species of the mold Penicillium may be divided into two classes: biosynthetic penicillins (those formed during the process of mold fermentation) and semisynthetic penicillins (those in which the structure of a chemical substance—6-aminopenicillanic acid—found in all penicillins is altered in various ways). Because it is possible to change the characteristics of the antibiotic, different types of penicillin are produced for different therapeutic purposes. Benzyl penicillin, or penicillin G, is the only naturally occurring penicillin that is still used clinically. Because of its poor stability in acid, much of penicillin G is broken down as it passes through the stomach; as a result of this characteristic, it must be given by intramuscular injection, which limits its usefulness. Some of the semi synthetic penicillins are more acid-stable and so may be given as oral medication.

Plate showing inhibition by Penicillin Penicillium spores



All penicillins work in the same way, namely, by inhibiting the bacterial enzymes responsible for cell-wall synthesis and activating other enzymes to break down the organisms' protective walls; therefore, they are not effective against microorganisms that do not produce cell walls. Bacteria, unlike animal cells, have a cell wall surrounding a cytoplasmic membrane. Production of the cell wall involves the partial assembly of wall components inside the cell, transport of these structures through the cell membrane to the growing wall, assembly into the wall, and finally cross-linking of the strands of wall material.

Antibiotics that inhibit the synthesis of a cell wall have a specific effect on one or another phase. The result is an alteration in the cell wall and in the shape of the organism and the eventual death of the bacterium.

Some strains of previously susceptible bacteria have developed a specific resistance to penicillin; these bacteria either produce penicillinases, enzymes that disrupt the internal structure of penicillin and thus destroy the antimicrobial action of the drug, or they lack cell-wall receptors for penicillin, greatly reducing the drug's ability to enter bacterial cells.

Among the bacteria sensitive to penicillin are those that cause throat infections, pneumonia, spinal meningitis, gas gangrene, diphtheria, syphilis, and gonorrhea. The chief side effects of penicillin are allergic or hypersensitivity reactions, including skin rashes, hives, swelling, and anaphylaxis, or allergic shock. Milder symptoms may be treated with corticosteroids but usually are prevented by switching to alternative medications; anaphylactic shock, which can occur in previously sensitized individuals within seconds or minutes, may require immediate administration of epinephrine to end the life-threatening process.

The penicillins and cephalosporins both have a unique structure, a β-lactam ring that is responsible for their antibacterial activity. The β-lactam ring interacts with proteins in the cell responsible for the final step in the assembly of the cell wall. Thus, the mechanism of action is identical for both antibiotics; however, the basic chemical structure of the penicillins and cephalosporins differs in other respects, resulting in some difference in pharmacokinetics and the spectrum of antimicrobial activity.

The penicillins can be divided into two groups: the naturally occurring penicillins (penicillin G, penicillin V, and benzathine penicillin) and the semisynthetic penicillins. The semisynthetic penicillins are produced by growing the mold Penicillium under conditions whereby only the basic molecule (6-aminopenicillanic acid) is produced. By adding certain chemical groups to this molecule, several different semisynthetic penicillins are produced that vary in resistance to degradation by β-lactamase (penicillinase), an enzyme that specifically breaks the β-lactam ring, thereby inactivating the antibiotic. In addition, the antimicrobial spectrum of activity and pharmacological properties of the natural penicillins can be changed and improved by these chemical modifications.

The naturally occurring penicillins are important chemotherapeutic agents. Even after 40 years of use they are still the drugs of choice for treating streptococcal sore throat, tonsillitis, pneumococcal pneumonia, endocarditis caused by some streptococci, syphilis, gonorrhea, meningococcal infections, and infections caused by some anaerobic organisms. Several microorganisms, most notably the staphylococci, developed resistance to the naturally occurring penicillins, which led to the production of the penicillinase-resistant penicillins (methicillin, oxacillin, nafcillin, cloxacillin, and dicloxacillin).

To extend the usefulness of the penicillins to the treatment of infections caused by Gram-negative rods, the broad-spectrum penicillins (ampicillin, amoxicillin, carbenicillin, and ticarcillin) were developed. These penicillins are sensitive to penicillinase, but they are useful in treating urinary tract infections caused by gram-negative rods as well as in treating typhoid and enteric fevers.

The penicillins are the safest of all antibiotics. The major adverse reaction associated with their use is hypersensitivity, with reactions ranging from a rash to bronchospasm and anaphylaxis. The more serious reactions are uncommon.

Commercial production of Penicillin: Penicillin G, one of the most active and widely used forms, is manufactured commercially using *Penicillium chrysogenum.* The process is carried out in stainless steel fermenters of l0 000 dm^3 capacity. The fermenter is steam sterilised and loaded with sterilised growth medium (corn steep liquor) containing lactose, amino acids, mineral salts and other substances. (Phenylethanoic acid, a metabolic intermediate, is also added, to increase the yield). An inoculum of strongly growing hyphae is added. Both glucose and nitrate are added periodically. The pH requires adjustment from time to time, to neutralise ammonia produced by the fungus. Temperature is set at first to give the maximum growth rate and then altered to favour penicillin synthesis. The fermenter is continuously stirred and sterile air blown in. An external cooling jacket is used for temperature control. Penicillin is a secondary metabolite, produced in large quantities only towards the end of the growth period of the fungus therefore it is essential for all of the mycelium to reach peak growth at the same time. This is why batch fermentation, rather than a continuous process, is appropriate for penicillin manufacture. After about 160-200 hours, the broth is filtered. Penicillin passes through in the filtrate which is further processed to crystallize the product

Cephalosporin: The cephalosporins are produced by *Cephalosporium acremonium*. Modification of the basic molecule (7-aminocephalosporanic acid) has resulted in three generations of cephalosporins.The first-generation cephalosporins (cefazolin, cephalothin, and cephapirin) have a range of antimicrobial activity similar to the broad-spectrum penicillins. The second-generation cephalosporins (cefamandole, cefonicid, cefotetan, cefoxitin, and cefuroxime) have greater β-lactamase stability than the earlier cephalosporins, and their antibacterial spectrum has been extended to include greater activity against additional species of gram-negative rods. They have decreased activity, however, against gram-positive bacteria. Like the penicillins, the cephalosporins are relatively nontoxic. Because the structure of the cephalosporins is similar to that of penicillin, hypersensitivity reactions can occur in penicillin-hypersensitive patients.

Bacitracin is produced by a special strain of *Bacillus subtilis*. Because of its toxicity its use is limited to the topical treatment of skin infections caused by streptococci and staphlococci and for eye and ear infections. Vancomycin, an antibiotic produced by Streptomyces orientalis, is poorly absorbed from the gastrointestinal tract and is usually given by intravenous injection. It is an excellent antibiotic for the treatment of serious staphylococcal infections caused by strains resistant to the various penicillins.

The **aminoglycosides** (streptomycin; neomycin; paromomycin; kanamycin and its derivative, amikacin; tobramycin; netilmicin; and spectinomycin) are produced by ***Streptomyces*** species. Gentamicin is produced by the molds *Micromonospora purpurea* and *M. echinospora*. All of the aminoglycosides inhibit protein synthesis, although spectinomycin, which has a different structure, does so by a mechanism different from the other aminoglycosides. The aminoglycosides are poorly absorbed from the gastrointestinal tract, so, with some exceptions, they are given by intramuscular injection. Neomycin is toxic and is used topically. Because it is poorly absorbed from the gastrointestinal tract, paromomycin is used in the treatment of protozoan infections of the intestinal tract.

Streptomycin was the first of the aminoglycosides to be discovered and the second antibiotic used in chemotherapy. One of its more important uses had been as part of the combined therapy for tuberculosis. It still has some use in combination with penicillin for treating infections of heart valves (endocarditis) and with tetracycline in the treatment of plague, tularemia, and brucellosis.

Kanamycin is used in the treatment of septicemia (blood poisoning), meningitis, and urinary tract infections caused by gram-negative bacteria. Because many organisms are resistant to its effects, however, kanamycin is now being replaced by other drugs. Gentamicin, tobramycin, netilmicin, and amikacin are similar in their range of antimicrobial activity. They are effective against infections caused by staphylococci and gram-negative bacteria, including *Pseudomonas aeruginosa.*

The major problem with the aminoglycosides is that the margin of safety between a toxic and a therapeutic dose is narrow. Nephrotoxicity (harmful to kidney cells) and ototoxicity (harmful to the eighth cranial nerve of the organs of hearing and balance) are frequent, and the risk of these reactions increases with age and with preexisting renal diseases or hearing loss. Spectinomycin does not have the serious toxicity associated with the other aminoglycosides. It is used solely in treating gonorrhea in persons who are hypersensitive to penicillin or in those with gonococci organisms resistant to penicillin.

Tetracyclines have a common structure but differ from each other by the presence or absence of chloride, methyl, and hydroxyl groups. Although these modifications do not change their broad-spectrum antimicrobial activity, they do affect pharmacological properties such as half-life in serum and protein-binding ability in serum. The tetracyclines all have the same antimicrobial spectrum, although there are some differences in sensitivity of the microorganisms to the various types of tetracyclines. They inhibit protein synthesis in both bacterial and animal cells. Bacteria have a system that allows tetracyclines to be transported into the cell, whereas animal cells do not; animal cells therefore are spared the effects of tetracycline on protein synthesis.

All tetracyclines are absorbed from the gastrointestinal tract after oral administration, and most can be given intravenously or intramuscularly. Because calcium, magnesium, aluminum, and iron form insoluble products with most tetracyclines, they cannot be given simultaneously with substances containing these minerals (e.g., milk). They are the drugs of choice in the treatment of cholera, rickettsial infections, relapsing fever, trachoma (a chronic infection involving the eye), psittacosis (a disease transmitted by certain birds), brucellosis, tularemia, and respiratory infections.

Tetracyclines are also used for acne vulgaris. Because not all of the orally administered tetracycline is absorbed from the gastrointestinal tract, the bacterial population of the intestine can become resistant to tetracyclines, resulting in overgrowth (suprainfection) of resistant organisms. Complexes between tetracyclines and calcium can cause staining of teeth and retardation of bone growth in young children or in the newborn if tetracyclines are taken by the mother after the fourth month of pregnancy. Tetracycline can also cause photosensitivity in patients exposed to sunlight.

Chloramphenicol now is synthesized chemically, but originally it was isolated from cultures of the bacterium *Streptomyces venezuelae*. It is administered either orally or parenterally, but since it is readily absorbed from the gastrointestinal tract, parenteral administration is reserved for serious infections. It is a broad-spectrum antibiotic used in the treatment of typhoid fever and for infections caused by microorganisms resistant to penicillin. Because newborns, particularly the premature newborn, cannot metabolize chloramphenicol, high levels accumulate and can cause inadequate oxygenation, the “gray syndrome.” The most serious adverse effect is a toxic decrease in bone-marrow activity and aplastic anaemia.

Erythromycin is produced by *Streptomyces erythreus*. It is usually administered orally, but it can be given parenterally. Although erythromycin has relatively few primary uses, it is valuable in treating pharyngitis and pneumonia caused by streptococci in persons sensitive to penicillin. It is also used in treating pneumonias caused either by Mycoplasma species or by the organism causing Legionnaire's disease; and it is used in treating pharyngeal carriers of the bacillus responsible for diphtheria.

Clindamycin is a derivative of lincomycin that has better microbial activity and rate of gastrointestinal absorption. As a result, lincomycin has limited use. Clindamycin is active against staphylococci, some streptococci, and anaerobic bacteria. Because it has been associated with pseudomembranous colitis (inflammation of the small intestine and the colon), it must be used with caution. Other antibiotics, however, can cause an identical colitis.

The **polymyxins** are produced by *Bacillus polymyxa* and are designated as polymyxin A through E. Two of these, polymyxin B and polymyxin E (colistin), are useful in treating infection. Polymyxins B and E are polypeptide antibiotics with an affinity for phospholipids (important elements in cell membranes). Polymyxins accumulate in the cell membrane of bacteria and affect selective permeability. They also react with and affect the membranes of animal cells, resulting in kidney damage and neurotoxicity. Because they are not well absorbed from the gastrointestinal tract, oral administration is occasionally used for the treatment of diarrhea. Polymyxins can be administered by intramuscular injection. They are used primarily in treating infections caused by Pseudomonas aeruginosa, but they are also used topically for the treatment of eye and ear infections. The availability of other excellent antibiotics limits their use. Rifampin, a semisynthetic agent derived from a rifamycin produced by *Streptomyces mediterranei*, inhibits RNA synthesis. It is absorbed from the gastrointestinal tract, penetrates tissue well, including the lung, and is used in the treatment of tuberculosis. Rifampin administration is associated with several side effects, mostly gastrointestinal in nature. The urine, feces, saliva, sweat, and tears can be red-orange in colour.

Sterilization-Principles-methods

The term control refers to reduction in number and activity of total microflora. The reasons for the control are

1. To prevent contamination or growth of undesirable microorganisms.

2. To prevent transmission of disease and infection

3. to prevent spoilage of material by microorganisms controlled by physical agents, physical processes or chemical agents

Fundamentals of control:

The term death is defined as irreversible loss of ability to reproduce

Physical agents: 1. Temperature (High&Low)

2. Dessication

3. Radiation

4.Filtration

1.High temperature: Organisms are from psychrophilic to thermophilic range. They have an optimum. maximum and minimum temperature.The temperature above maximum will kill the organisms and below minimum will cause stasis.High temperature with high moisture is an efficient method. Moist heat coagulate the protein which is rapid than dry heat which destroy their chemical constituents. Thermal Death time: Refers to the shortest time period to kill a bacterial suspension at a prescribed temperature under special conditions Decimal reduction time: Time in minutesto reduce the population by 90% Moist heat: Heat in the form of saturated steam under pressure. It has the advantage of rapid heating, penetration which helps in coagulation of proteins. Growth media,old cultures are sterilized by this method. Autoclave is the equipment where the saturated steam with pressure is developed and media are kept for 20 minutes at 15lbs pressure.

Fractional sterilization: Developed by John Tyndall in this method, materials are steamed for 3 consecutive days thereby the germinated spores are also completely destroyed.This is also called Tyndallization or intermittent sterilization.

Dry heat: Glasswares,oils,powders are sterilized in Hot air oven for 2 hours at 160 degree celsius.

Incineration: by burning eg) carcasses ,inoculation needle

Low temp; Culture are stored for a long period at 4-7 degree celsius.

Radiation: Energy transmitted through space in a variety of forms is ionization UV light-150-3900 Angstrom. 2650 Angstrom has the highest bactericidal efficiency. Mode of action: UV absorbed by nucleic acids. DNA replication can be inhibited and mutation can result. Other rays are x-rays, gamma rays

Filtration: Seitz filters, Sintered glass filter and cellulose membrane filter.Here the pore size of the filter is smaller than bacterial cell. So bacteria retained on the filter and clear solution passes through.

HEPA filters: To deliver clean air to an enclosure viz., Laminar Flow chamber

Chemical agents:

Characteristics:

1.Easily available. 2.Non toxic 3. Antimicrobial at low conc.4. Non corrosive 5.stability 6.cheaper 7.capacity to penetrate 8.deodorising ability

Important terms:

Sterilization: Process of destroying all forms of microbial life.

Disinfectant: Kills growing forms. but not resistant spore forms. Used on inanimate objects.

Antiseptic: Prevent growth by inhibiting the metabolism. applied to body.

Bactericide kills bacteria whereas bacteriostatic temporarily arrest the growth of bacteria.

Major compounds:

1. Phenolic compounds 2. Alcohols 3. Halogens 4. Heavy metal compounds

5. Dyes 6.Detergents 7.aldehydes

Phenols: Joseph Lister used phenol in 1880s to redfuce infection in surgical incision.Later he sorayed in the operation room area. 5% aqueous phenol rapidly kills the vegetative cells. Derivatives of phenol used in many preparations of disinfectants.

Mode: Precipitation of cell protein, inactivate the enzymes

Alcohols: 90% effective

application: used on skin, cleaning clinical thermometers

mode: Protein denaturation,solvent of lipids

Halogens: Iodine: Used in the form of tincture of iodine.

Use: disinfection of skin

Mode: oxidize essential metabolic compounds such as proteins

Chlorines: either in the form of gas or in certain chemical combination and used as disinfecting agent. Purification of municipal water supplies Eg) calcium hypochlorite, Sodium hypochlorite

Heavy metals: Mercury,silver and copper

Mode: combine with cellular protein and inactivate them.

Antibiotics and chemoterapeutic agents: Treatment of a disease with chemical is called chemotheraphy. Naturally synthesized materials are called antibiotics.

Characteristics: 1.Destroy parasite without injuring cell

2. Leave no host defence mechanism unaltered

Eg) sulfanimides-Paul Ehrlich found this effective against wide variety of parasites.

Antibiotics: Substance of microbial origin which can kill or inhibit other microbes at lower concentration.

Mode of action:

1.by inhibition of cell wall synthesis

2.by inhibition of enzyme systems

3.by inhibition of nucleic acid and protein synthesis

4.by damaging cytoplasmic membrane

UNIT – II

BACTERIAL BIOMASS

Introduction

To keep up with world population, projected at about 10 billion by the twenty-first century, food production will have to increase fivefold. Such an increase can be brought about increasing either the area planted or the yield per unit area. Most of the agricultural land today is being rapidly diverted for other uses, limiting further the area that can be used for increasing food production. Genetic improvement of crop varieties is an alternative with its own limitations.

The term *single-cell* protein (SCP), coined to describe unconventional protein sources like those obtained from monocellular bacteria, algae, and yeasts, has been replaced by the term *microbial biomass protein* (MBP), to allow inclusion of higher fungi as unconventional sources of protein. *Biomass* refers to the organic cell substance of a living organism. Production of microbial biomass to satisfy world food demand, especially demand from the developing world, appears to be the most attractive alternative to date for at least six reasons:

1. Microbes require less time to grow than crops.

2. Production can be carried out in fermentors without requiring too much land or technical control.

3. Microbial biomass production processes are independent of the vagaries of nature.

4. Microbial biomass processes can be genetically manipulated with ease to improve production processes and product quality.

5. These processes have high protein contents (35-60%).

6. The nutritional value of MBPs is as good as that of other conventional foods rich in protein.

The use of microorganisms for human consumption dates back at least 6000 years to the time when *Saccharomyces* spp. were used in the production of bread, beer, and wine.

The Aztecs consumed blue-green algae, which served as a major source of protein in their diet. The Egyptians and Greeks consumed fermented milk and cheeses produced by species *of Streptococcus* and *Lactobacillus.*

Molds have been used in the production of oriental fermented foods. Although baker's yeast has been produced on a large scale since the early 1900s, the first move toward growing microbes for food was made when the yeast *Candida utilis* grown on sulfite waste liquor (SWL), a by-product of the pulp and paper industry, was produced for use as a human and animal food supplement in Germany during the Second World War. In the late 1950s, commercial production of MBP on hydrocarbons was attempted. The British Petroleum Company was the first to enter this field, followed later by others. In the 1970s Shell developed a process for SCP production using methane as a substrate. Since then there have been many new technological developments in this area of research. In this chapter, "biomass" includes the cells of other microorganisms besides bacteria for the convenient treatment of the subject.

Microorganisms for the Production of Biomass

Numerous species of bacteria, and some species of actinomycetes, fungi, and algae, have been proposed as attractive candidates for the production of biomass protein. These organisms are capable of utilizing a wide range of carbon and energy sources for their propagation. Table 5.14 lists some organisms and substrates used in these processes. Desirable characteristics include genetic stablility, high substrate conversion, high specific growth rate and productivity, easy separation, good quality and composition, and absence of pathogenicity.

Bacteria are able to use a wide variety of substrates ranging from simple sugars and complex carbohydrates to petrochemical products. The ability to utilize methane as a carbon source is a property almost exclusively present in bacteria. These bacteria, called the *methanotrophs,* may be found in anaerobic environments such as those observed in waste digestion processes. *Methylomonas methanooxidans, Methylophilus methylotrophus,* and *Methylococcus capsulatus* are examples of bacteria being used for the production of biomass protein from methane. These organisms, as well as action mycetes (e.g., *Streptomyces* spp.), yeasts (e.g., *Candida* spp., *Hansenula* spp., *Torulopsis* spp.), and fungi (e.g., *Trichoderma* spp.), are also able to grow on methanol.

Compared with the methylotrophic bacteria, however, the yeast strains assimilate methanol with a poor efficiency of conversion, higher oxygen demand, greater reaction heat, lower fermentation temperature, and lower protein content. Bacteria in general have faster growth rates (generation times ≈ 20-120 min) than yeasts, molds, and fungi (2-16 h or more). The cultivation of higher fungi for use as part of the normal human diet is not uncommon. The *sporophores* (fruiting bodies) of the basidiomycetic species *Agaricus bisporus* are common ingredients in many of our food items.

SOURCE ORGANISMS AND SUBSTRATE USED FOR THE PRODUCTION OF BACTERIAL BIOMASS

Organism	**Carbon or Energy Source**
Bacteria	
Cellulomonas spp.	Bagasse
Alcaligenes spp.	
Methylophilus methylotrophus	Methanol
Methylococcus capsulatus	Methane
Yeast	
Candida utilis	Ethanol, sulfite waste liquor
Candida lipolytica	n-Alkanes
Kluyveromyces fragilis	Cheese whey
Saccharomyces cerevisiae	Molasses
Mold and Higher Fungi	
Cephalosporium eichorniae	Cassava starch
Paecilomyces varioti	Sulfite waste liquor
Penicillium cyclopium	Cheese whey (lactose)

Chaetomium cellulolyticum	Agriculture and forestry waste
Algae	
Scenedesmus acutus	CO_2, sunlight
Spirulina maxima	CO_2, HCO_3, CO_3 sunlight

The cultivation of higher fungi for use as a source of protein offers many advantages. However, the protein content is in general unfavorable and nutritional quality is poor compared to yeasts or bacteria. The productivity of fungal biomass production processes is also lower, in the range of 3^ kg/m^3. Many fungi are known to produce a range of undesirable metabolites (e.g., oxalic acid, mycotoxins).

Most bacterial SCP processes use pure cultures, but mixed cultures of *Cellulomonas* spp. and *Alcaligenes faecalis* have been shown to be more effective for the use of cellulose. *A. faecalis* in this mixed culture system is able to utilize the soluble sugars produced by the *Cellulomonas* spp., which have strong cellulose activity but do not possess p-glucosidase activity strong enough to be able to bring about complete utilization of cellulose.

Another example of mixed cultures is the production of bacterial biomass from methane using species of *Pseudomonas, Hyphomicrobium, Acinetobacter,* and *Flavobacterim.* When grown together, they had a higher growth rate, higher yield coefficient, and less foaming than pure cultures. Mixed cultures of yeast and fungi have also been used. For instance *Saccharomyces cerevisiae* and *Trichoderma viride* grown on cassava were found to produce a better protein content than the yeast alone. In the production of biomass from methane using a methane assimilating strain, *Methylococcus capsulatus,* addition of *Pseudomonas, Nocardia,* and *Moraxella* had a stabilizing effect, which was attributed to the ability of the auxiliary cultures to assimilate toxic metabolites of the main strain.

Raw Materials for the Production of Biomass

The raw materials required for the growth and metabolism of microorganisms include a carbon or energy source, a nitrogen source, and supplementary nutrients.

The carbon or energy sources that have been considered to be suitable for the production of microbial biomass protein fall into two broad categories: substrates from renewable resources and substrates from nonrenewable resources

Substrates from Renewable Resources

Substrates obtained from the renewable resources in the agricultural, forestry, and food processing sectors are both cheaply available and abundant. Lignocellulosic materials, widely available in agricultural or forestry product residues, are the most abundant resources (estimated at 100 billion tons/year). These substrates are composed of simple sugars and complex carbohydrates. The simple sugars are the hexoses (e.g., glucose, galactose, fructose, mannose), the pentoses (e.g., xylose), and the disaccharides (e.g., lactose, sucrose). The complex carbohydrates are starch, hemicellulose, and cellulose. The products constituting these compounds include potatoes, cassava, whey, sulfite waste liquor, and molasses. The chief disadvantage of these products is the high cost of collecting and transporting sufficient quantities to operate a processing facility large enough to be viable economically; such costs can be prohibitive in less-developed countries. Also, many of the substrates are available only seasonally, which means that in many regions SCP production facilities could operate during only a portion of the year.

SUBSTRATES FOR MICROBIAL PROTEIN PRODUCTION

Material	**Availability**	**Pretreatment**	**Yield (g/g substrate)**	**Use**
Saccharides				
Molasses	Seasonal	Simple	0.25-0.33	Animal feed
Whey	Year-round	None	0.03	Fractionation
Sulfite waste	Year-round	Simple	0.008	Animal feed
Potato waste	Seasonal	None	0.5	Animal feed
Fruit	Seasonal	None/simple	0.03	
Polysaccharides				

Starch	Seasonal	Hydrolysis	0.5-0.6	Food
Cellulose	Year-round	Hydrolysis	0.03	Fuel/animal feed
Hydrocarbons				
Methane	Year-round	None	0.3-1.4	Fuel/chemical feedstock
n-Paraffins	Year-round	Separation	1.0	Fuel/chemical feedstock
Alcohols	Year-round	None	0.25-0.5	Fuel
Methanol				
Ethanol	Year-round	None	0.6-0.7	Fuel
Propanol	Year-round	None	0.4	
Other				
Acetate/Malate		Dependent on source	0.35	

Source: Adapted from Davis (1974) and Lee (1991).

Yeasts such as *Endomyces vernalis* and *Saccharomyces cerevisiae* and fungi such as *Candida utilis* are able to utilize pentoses and may therefore be employed in the production of biomass protein from sulfite waste liquor, introduced in Section 5.5.1. The *Pekilo* process, the largest fungal biomass-based process, was developed in Finland at the Finnish Pulp and Paper Institute. It produces 15-16.5 tons dry weight of *Paecilomyces varioti* mycelium on SWL in 24 hours with a protein content of 55%. Similarly whey, a by-product of the cheese industry, may serve as a suitable substrate for the lactose-utilizing yeast *Kluyveromyces fragilis.* Both whey and sulfite waste liquor have very high biological oxygen demand (BOD), causing serious problems to the industrial sectors producing them because of the high cost of disposal. Disposal is expensive because the high BOD cannot be tolerated by the environment.

The complex carbohydrates may serve as substrates for organisms that are capable of producing the enzymes necessary for the breakdown of the large carbohydrate molecules to smaller, assimilable sugars.

Many fungal cultures, including *Trichoderma viride, Aspergillus niger,* and *Sporotrichum pulverulentum,* have the ability to do so and can therefore use these substrates directly. Yeasts, however, do not produce complex carbohydrate hydrolyzing enzymes and therefore are able to utilize these substrates only after pretreatment. The most common pretreatment methods, grinding and steam explosion, render the complex substrates available for utilization by yeasts. Bacterial species like *Cellulomonas, Bacillus,* and *Brevibacterium* have demonstrated the ability to produce protein on lignocellulosic waste materials. Since the 1950s there has been considerable interest in the large-scale cultivation of algae from the renewable resources, CO_2, and sunlight.

Algae can also be grown using artificial lighting or heterotrophically, in the dark, with organic carbon and energy sources. Single-celled or multicellular, filamentous algal cultures (e.g., *Chlorella, Scenedesmus* spp., *Spirulina maxima)* can utilize CO_2 as a source of carbon and energy. These phototrophic organisms, which also include phototrophic bacteria such as *Rhodopseudomonas,* are cultivated in flat trays containing medium supplemented with additional nutrients. The major disadvantage of growing algae is the requirement for vast stretches of land. Since the process also depends on the availability of light, it may be useful only in geographical regions where light is abundant. Another disadvantage is the high cost of harvesting algal cultures, which do not reach high cell densities because of limited capacities for the absorption of light. An attempt was made to grow the multicellular, filamentous *Hydrodictyon* on wastewater from the breeding offish, which were fed a diet of this algal species (Prave et al., 1987).

Substrates from Nonrenewable Resources

Nonrenewable petrochemical substrates include gas-oil and paraffins (ethane, propane, n-and isobutene). The normal paraffin process developed by the British Petroleum Company was based on the growth of Candida lipolytica on paraffin. During the 1950s these substrates were considered to be economically feasible for use in the production of biomass protein. By definition, however, these substrates were always limited in their availability, and after the hike in petroleum and natural gas prices in the early 1970s the plants ceased to be profitable, hence had to be shut down.

Licensing problems including toxicity, together with the increase in oil prices, stimulated efforts toward SCP production from methanol and ethanol.Several properties make methanol an attractive candidate for the use as substrate: it has a high solubility in water; it can be removed easily when the process is complete; it gives high productivity; and it lacks the explosivity hazards associated with other petroleum-based substrates. Ethanol can be whereas for functional applications, the functional behavior of the biomass ingredients in the food products has greater importance. With all three, however, the product must be free of toxins or other undesirable metabolites, heavy metals, and pathogens; it must have acceptable sensory properties, and a low viable cell count.Since "total nitrogen" includes such nonprotein nitrogenous substances as nucleic acids and gives no information about the amino acid profiles, it does not reflect true nutritional value. A comparison of the amino acid profiles of some biomass proteins with the reference protein established by the Food and Agriculture Organization (FAO) of the United Nations reveals that microbial proteins tend to be deficient in methionine. Among the different protein sources, bacterial proteins have higher levels of methionine than yeasts, fungi, and soybean (Table 5.16).

The best approximation of the nutritional value can be obtained by animal feeding studies. The parameters that are studied include determination *of protein digestibility, protein efficiency ratio* (PER), *biological value* (BV), and *feed conversion ratio* (kilograms of product consumed to kilograms of weight gained). From such studies, the blue-green alga *Spirulina* was shown to be a good source of protein in experiments with sexually maturing rats and was used as the sole source of protein in their diet (Contrezas et al., 1979).

COMPARATIVE ANALYSIS OF VARIOUS BIOMASS PRODUCTS

Biomass Products*	**Bacteria/ Methanol**	**Yeasts/ Paraffin**	**Yeasts/ Carbohydrates**	**Fungi/ Carbohydrates**	**Algae/ CO_2**
Crude protein, %	80	55-60	45-50	35-45	40-60
Nucleic acids, %	10-15	5-8	10	10	6
Fat %	8	9	2-5	2-5	5-9
Minerals, %	7-8	8	5-10	5-10	10-15

Selected amino acids, g/ 16 g nitrogen

Isoleucine	4.5	3	4.5	5	5-6
Alanine	7	6	6	6.5	
Leucine	7	5.5	6.5	7	8-9
Glycine	5.5	3	5	5	
Lysine	6	6.5	6.5	6.5	4-5
Phenylalanine	3.5	2.5	3.5	4	4-5
Methionine	2.5	2	1.5	2	2-3
Proline	3.5	2.5	3.5	4	
Threonine	4.5	3.5	5.5	4	5
Aspartic acid	9	8	8	9	
Tryptophan	1	0.5	1	1	1
Glutamic acid	10	9	10	2	
Valine	5	3.5	5	5	6-7
Tyrosine	3	3	3.5	3.5	5
Arginine	4.5	3.5	4.5	5	9-10
Histidine	2.5	2	3	2	1.8
Serine	3.5	3	3.5	4	

** Various biomass products that are produced by a given group of organisms when growing on a given carbon substrate (e.g., bacteria growing on methanol (bacteria/ methanol)).*

Although bacteria and molds have been consumed for many centuries, many of them have been shown to produce toxic substances. Moreover, the substrate used for production of the biomass may be toxic and may remain associated with the biomass after processing. Therefore, before a new product can be marketed, a number of tests must be carried out to determine the safety of the product. The U.S. Food and Drug Administration (FDA) and the Protein Evaluations Group of the FAO have developed guidelines for evaluating the safety of products in human and domestic livestock. The nutritional and toxicological testing process can cost millions of dollars, and ten years may elapse before approval is granted. Some of the common problems that may arise ire allergic skin reactions and digestive problems.

Many sources of microbial biomass protein are not digestible because many of these organisms produce compounds such as intracellular polymerized lipids and cellulose. In human feeding studies, consumption of certain MBPs has been shown to cause a wide range of gastrointestinal complaints, from relatively mild symptoms such as bulky stools and flatulence to the more serious symptoms of nausea, vomiting, and diarrhea (Litchfield, 1985). Other pathophysiological reactions noted included peeling skin from the palms of the hands and the soles of the feet.

Microorganisms have a high content of nucleic acids—bacterial cells, for example, contain 6-11% (dry weight basis). The estimated safe intake for a healthy adult is 2 g/day. Taking the example further, we note that human beings lack the enzyme uricase, which breaks down uric acid to allantoin. We note also that consumption of yeast as a source of protein would cause an increase in the serum uric acid levels. Uric acid, being only slightly soluble at physiological pH, may crystallize out in joints, causing gout as well as kidney stone formation. Methods described (Litchfield, 1985) to reduce the nucleic acid content to 1-2% of the tolerance level include (1) lowering the growth rate, thus minimizing the RNA content, (2) heat-shock incubation for degrading ribonucleases, (i.e., rapid heating of the culture to 64°C to inactivate the fungal proteases and allow the endogenous RNases to hydrolyze the disrupted ribosomal RNA, whereupon the 5' nucleotides produce diffuse into the culture broth and are thus eliminated), and (3) the use of alkali to extract and coagulate the nucleic acid.

The third method has also been demonstrated to improve the consistency, color, and odor of the Pekilo protein biomass when the alkali is neutralized with acid before the biomass is washed. However allowing the pH to drop below 6 caused reprecipitation of RNA onto the biomass. Livestock are able to tolerate higher levels of nucleic acid than humans. Human consumption of *Alcaligenes eutrophus* was found to cause gastrointestinal disturbances such as nausea and vomiting, while these effects were not observed in animal feeding studies.

Microbial biomass protein can also be used to impart to processed foods various characteristics such as flavor, fat and water binding, dispersing action, whipping and foaming action, or extrusion and spinning properties. There is no information regarding whether these proteins have any better functional properties than the conventionally used soymeal protein concentrates or isolates.

Also, since these proteins are still more expensive than the soymeal protein, there has been no attempt to replace the soy protein with MBP.

Economics and New Developments

A major economic constraint is the large working capital required for the bacterial biomass process. This is followed by the cost of the raw material, which is estimated to account for 50-70% of the cost of production. Table 5.17 compares five SCP processes with soymeal or fish meal in terms of costs and protein content. The cost of the raw material can be minimized by coupling the biomass protein. production process with another existing process whose waste products can be utilized as substrates for MBP production. Food-grade yeasts such as *Saccharomyces cere-visiae* and *Kluyveromyces fragilis* have a good position in the food ingredient markets, but yeasts cannot utilize lactose in whey or agricultural biomass (xylose), which are two promising substrates. Through the use of genetic engineering, the characteristic enzyme systems required can be introduced into yeasts, consequently enabling the use of less expensive, more abundant substrate, which will lead to a decrease in costs. The techniques of recombinant DNA technology have been employed to increase the efficiency of conversion of a process. *Methylophilus methylotrophus,* when grown on methanol and NH_3, is an important source of SCP. This organism lacks the enzyme glu-tamate dehydrogenase and instead uses the ATP-dependent glutamine synthetase/gluta-mate synthetase (GS/GOGAT) pathway for the assimilation of NH_3.

This results in wastage of methanol. Cloning and expression of the glutamate dehydrogenase gene from *E. coli* resulted in a 4.7% increase in the efficiency of the carbon conversion. The temperature at which a fermentation is carried out has a significant role in determining the economics of a process. The higher the operating temperature, the lower is the cooling cost. Therefore development of thermotolerant strains might help to reduce the cooling cost. With technological advances such as the development of cheaper recovery methods, processes that allow achievement of higher cell densities and productivities, and genetically engineered strains having better conversion efficiency and the ability to use cheaply available substrates, the economics of the process should begin to look more attractive.

ECONOMICS OF THE FIVE SCP PROCESSES VERSUS SOYMEAL AND FISH MEAL

Process	**Investment ($ x 10^6)**	**Unit Product Cost ($/ton)**	**Raw Materials Utilities ($/ton)**	**Protein Content (%)**	**Protein Cost ($/ton)**
Bagasse	78,204	1,098	465	60	1,830
Molasses	48,770	756	332	52	1,454
Methanol	77,200	1,264	618	71	1,780
Cassava		605*	212+	17	3,558
Sulfite liquor	14,000	340	290	50	688
Soymeal		150		49	315
Fish meal		330		60	530

** Raw materials assumed at 35% of total unit product cost.*

+ Based on raw materials only (utilities not included).

Source; *Author's compiled data; 1985 U.S.ollar amounts.*

SINGLE CELL PROTEINS

The term Single Cell Protein refers to a protein derived from micro-organisms. The term SCP refers to the dried cells of microorganisms such as algae, actinomycetes, bacteria, yeasts, molds, and higher fungi grown in large scale culture systems for use as protein sources in human foods or animal feeds.Although these microorganisms are grown primarily for their protein contents in SCP production process, microbial cells contain carbohydrates, lipids, vitamins, minerals and non-protein nitrogen materials such as nucleic acids. It was coined by Professor Wilson, to replace the less inviting microbial or bacterial protein or petroprotein (for cells grown specifically on petroleum). To overcome the protein malnutrition this SCP was used to produce proteins from plants such as oil beans or from animals such as fish. It was not subjected to vicissitudes of the weather and can be produced every minute of the year and micro-organisms have much more rapid growth than plants or animals. Furthermore, waste products can be turned into food in the production of SCP.

The disadvantages of this SCP are financial resources to develop the highly capital intensive fermentation industries involved and the SCP are the microorganisms that contain high levels of RNA and that its consumption could lead to uric acid accumulation, kidney stone formation and gout. Microorganisms began receiving attention as food worldwide from the early 1960's. Fermented milks and yoghurts which have been consumed till today have large amounts of bacteria and yeasts. The blue green algae, Spirulina has been eaten for centuries.

SUBSTRATES FOR SINGLE CELL PROTEIN PRODUCTION:

A wide variety of substrates have been used for SCP production and these include the hydrocarbons especially gaseous hydrocarbons. Among the **gaseous hydrocarbons**, methane is the most widely studied as a source of SCP. Other gases include propane and butane. SCP from methane has used continuous cultures and a mixed population of microorganisms.The advantages of mixed methane are higher growth rates, higher yield co-efficient, greater stability resistance to contaminations and reduction in foam production.The major source of **liquid hydrocarbons**, is crude petroleum.This was first studied as a source of microbial vitamins and lipids. **Alcohols** such as methanol and ethanol were used as alternatives to petroleum substrates.

Due to the high prices of petroleum products it is unlikely that petroleum-based substrates such as methanol, ethanol, gas oil etc., will be used in future. A large number of reports of SCP from waste material such as plant/wood wastes (cellulose containing materials such as corn cobs, plant stems, leaves, stalks, husks), starch-wastes (starch containing waste from rice, potato or cassava manufacturing industry), dairy wastes (Whey, a by-product of the diary industry), wastes from chemical industries (*C.lipolutica* or *Trichosporon cutaneum* in oxanone water)

Starchy materials, such as potato processing waste, must be converted to mono- and disaccharides to be suitable as substrates for SCP production. The Swedish Sugar Corporation developed the Symba process for treatment of wastes that contain starch, such as those from potato and rice processing. Two organisms used are Saccharomycopsis Jihuligera, which produces a- and **P**amylases for hydrolysis of starch to glucose and maltose. and C. utilis for utilizing these sugars as a substrate for growth . This process was operated on a pilot plant scale to produce 40 to 100 kilograms of dry yeast per day, but its usefulness is limited by the intermittent availability of the waste stream from potato processing operations.

Alternatively, starch can be hydrolyzed by a combination of a-amylase and amyloglucos~dase(glucoamylase) as used in converting starchy materials for ethanol production by fermentation. In addition to the carbon and energy source, microorganisms require sources of nitrogen, phosphorus, and mineral nutrients, and may require supplemental nutrients such as vitamins. Suitable nitrogen sources for SCP production are ammonia, ammonium salts, nitrates, urea, and organic nitrogen sources such as protein hydrolyzates. It is important to adjust the supply of the nitrogen source so that a ratio of 10: 1 or less for carbon to nitrogen can be maintained in the medium during growth to minimize the accumulation of lipids or storage substances, such as poly-p-hydroxybutyrate, and to favor high protein contents in the cell. The phosphorus source for SCP production is usually supplied as either phosphoric acid or soluble phosphates; a food- or feed-grade source of phosphorus, which is low in arsenic, fluoride, or heavy metals, should be used.

It is apparent that large quantities of process water are required for SCP production,including medium preparation,cell washing, cleanup, and steam generation.For example, an estimated water requirement for producing bacterial SCP from methanol is in the range of 45.5 milllion liters per 100,000 metric tons per year of production

Table 1. Selected raw materials used as carbon and energy sources in single-cell protein processes.

Raw material	Process type and scale*	Organism	Producer or developer
	Algal, 2 metric tons per day†	*Chlorella* sp.	Taiwan Chlorella Manufacture Co. Ltd., Taipei
CO_2	Photosynthetic		
Cane syrup, molasses (sucrose)	Nonphotosynthetic		
CO_2 or $NaHCO_3$-Na_2CO_3	Algal, 320 metric tons per year;‡ photosynthetic	*Spirulina maxima*	Sosa Texcoco, S.A., Mexico City
Methanol	Bacterial		
	70,000 metric tons per year	*Methylophilus methylotrophus*	Imperial Chemical Industries, Billingham
	1000 metric tons per year	*Methylomonas clara*	Hoechst-Uhde, Frankfurt, West Germany
Ethanol	Yeast, 7500 short tons per year	*Candida utilis* (Torula)	Pure Culture Products, Hutchinson, Minnesota
n-Alkanes, wood hydrolyzates	Yeast (several plants), 20,000 to 40,000 metric tons per year	*Candida* sp.	All-Union Research Institute of Protein Biosynthesis, U.S.S.R.
Sulfite waste liquor	Yeast, 15 short tons per day	*Candida utilis*	Rhinelander Paper Corp., Rhinelander, Wisconsin
	Mold, 10,000 metric tons per year	*Paecilomyces varioti*	Pekilo Process, Finnish Pulp and Paper Research Institute, Jamsankoski, Finland
Glucose	Mold, 50 to 100 metric tons per year	*Fusarium graminearum*	Rank Hovis MacDougall Research Limited, High Wycombe, U.K.
Cheese whey (lactose)	Yeast, 5000 short tons per year	*Kluyveromyces fragilis*	Amber Laboratories, Juneau, Wisconsin
	Mold, 300 metric tons per year	*Penicillium cyclopium*	Heurty, S. A., France

PRODUCTION OF SCP

Production of SCP involves following basic steps:

(i) Preparation of suitable medium with suitable carbon source;

(ii) Prevention of contamination of medium and the plant;

(iii) Production of the desired micro organism;

(iv) Separation of microbial biomass and its processing.

MICROORGANISMS USED IN SCP PRODUCITON:

The heterotrophic microorganisms currently used are bacteria, actinomycetes and fungi. The organism to be used in SCP production should have the following properties:

(a) Absence of pathogenicity and toxicity

(b) Protein quality and content

(c) Digestibility and organoleptic qualities

(d) Adaptability to unusual environmental conditions

PROCESS CHARACTERISTICS

The type of process (batch or continuous),growth rate, sterility requirements, type offermentor or bioreactor, extent of feedstock utilization and yield, temperature, pH**,** and methods used for product recovery all are important factors in determining the economic viability of SCP process (Table 2).

The composition of the cell product will vary with different algal species. Algae can be grown either in batch tanks or semicontinuous ponds operated on a fill and draw principle

Table 2. Operating characteristics of selected single-cell protein processes.

Item	Process			
	Algal *Spirulina maxima*	Bacterial *Methylophilus methylotrophus* (methanol)	Yeast *Candida utilis* (ethanol)	Mold *Paecilomyces varioti* (sulfite waste liquor)
Type of process	Batch or semicontinuous	Continuous	Continuous or batch	Continuous
Sterility	Nonaseptic	Aseptic	Aseptic	Nonaseptic
Fermentor	Ponds	Airlift	Agitated	Agitated
Feedstock utilization	Partially or fully utilized	Fully utilized	Fully utilized	Partially utilized
Temperature (°C)	Ambient	35 to 42	30 to 40	38 to 39
*p*H	9 to 11	6.0 to 7.0	4.6	4.5 to 4.7
Product recovery	Filtration	Agglomeration and centrifugation	Centrifugation	Filtration

Photosynthetic organisms : Both algae and photosynthetic bacteria have been used for SCP production. The photosynthetic production of SCP by ***Chlorella*** species can be represented by the following typical equation.

6.14 C02 + 3.65 H20 + NH3 % C6 14H10.3024 N + 6.85 0 2

Photosynthetic bacteria such as ***Rhodopseudomonas capsulata*** have been grown in Japan, with sewage or industrial waste as substrates. Generally, these organisms grow in mixed culture with aerobic, heterotrophic, and nitrogen-fixing bacteria.

Nonphotosynthetic organisms. Actinomycetes, nonphotosynthetic bacteria, molds, yeasts, and higher fungi all require aerobic conditions for growth in SCP processes.

Many species of bacteria have been investigated for use in SCP processes because of their short generation times (20 to 30 minutes) and their ability to use a variety of raw materials ranging from carbohydrates to gaseous and liquid hydrocarbons and petrochemicals.Figure 1shows a schematic diagram for bacterial SCP production from methanol.An example of a commercial-scale SCP process based on methanol now in operation is that developed by Imperial Chemical Industries, Ltd., in the United Kingdom for growing ***Methylophilus methylotrophus.*** The conversion of methanol to the SCP product is represented by the equation

$$1.72\ CH_3OH + 0.23\ NH_3 + 1.51\ O_2 \rightarrow$$
$$1.0\ CH_{1.68}O_{0.36}N_{0.22} +$$
$$0.72\ CO_2 + 2.94\ H_2O$$

This process has been operated intermittently over the past year at 6000 metric tons per month. The organism is grown continuously under aseptic conditions (Table 2). A special "pressure cycle"

airlift type fermentor is used. The process is operated at 35' to 42°C to minimize cooling cost, since the growth of these organisms is highly exothermic. An unusual feature of the process is the initial separation of the cells from the production medium by agglomeration in the aqueous growth medium so that a higher solids slurry can be fed to the centrifuges than is normally practiced in

most SCP processes. Purified C02 is sold as a by-product. Relatively high specific growth rates of 0.50 hour-' and cell yields exceeding 0.50 gram per gram of substrate are reported for this process.

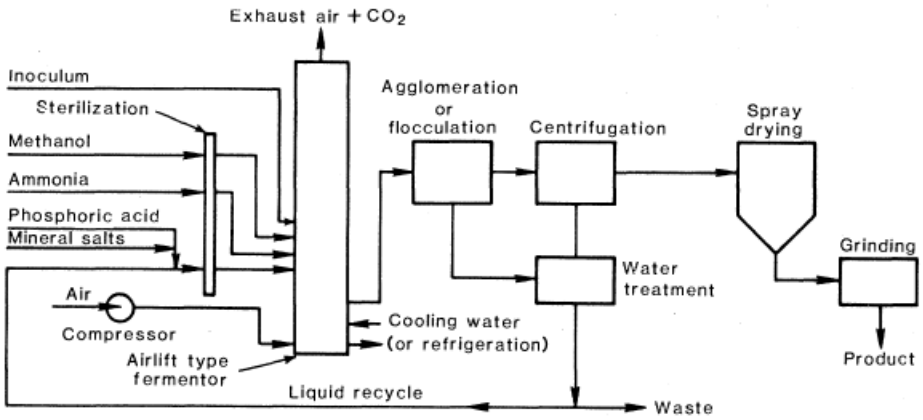


Fig. 1. Schematic diagram of a typical process for making single-cell protein from methanol (5).

Product Quality and Safety

Single-cell protein products can be used as (i) protein supplements in human foods, (ii) functional food ingredients to provide, for example, flavor, fat and water binding, dispersing action, whipping

and foaming action, and extrusion and spinning characteristics, and (iii)protein supplements for livestock feeding

SCP has strong opposition especially because of the following reasons:

- Minimata disease –due to the consumption of mercury from sea foods
- Carcinogenic compounds in petroleum-grown SCP
- SCP leds to consumption of high levels of nucleic acid. Man has lost the enzyme uricase which oxidizes uric acid to the soluble and excretable allantoin. High levels of nucleic acid leads to higher levels of uric acid occur in blood plasma. Owing to the low solubility of uric acid, uricates may be deposited in various tissues in the body including the kidney and joints when the disease known as kidney stones and gout may result.

MARKET CONSIDERATIONS FOR SCP

Establishing markets or maintaining existing markets for SCP products for animal feed applications depends on their price and feeding performance in broiler chicken, turkey, laying hen, or swine rations as compared with existing protein feedstuffs such as soybean meal and fish meal.In human foods, flavor and texture, in addition to nutritional value of SCP products, are important determinants of acceptability. At the present time, the major market for food-grade SCP productsis for functional uses in foods. For example, yeast protein autolyzates and hydrolyzates have been used as food flavoring for many years.

Torula yeast products are being sold as functional food additives in processed meats and bakery products in the United States.

NUTRITIONAL VALUE OF SCP

- Depends on the composition of microbial cells used especially their protein, aminoacid, vitamin and mineral content.
- Depends on the conditions of growth of the organism
- SCP from bacteria and yeasts is deficient in methionine
- Molds are deficient in glycine and methionine

PROSPECTS

Table summarizes some of the current research and development efforts that may lead to significant improvements in SCP processes. These range from strain improvement, including genetically engineered cultures such as the glutamic dehydrogenase recombinants developed by ICI in the United Kingdom, to improved methods for protein isolation and cell harvesting, process monitoring, and computer control of production .However, the impact of these developments on the future economic viability of SCP processes remains to be seen.

Table 5. Improvements in single-cell protein production.

Item	Example
Strain improvement	Mutants of *S. cerevisiae* forming enlarged cells for improved recovery
Genetically engineered cultures	Cloning of genes for higher amino acid contents in methanol-utilizing bacteria
	Improved NH_3 utilization by transfer of glutamic dehydrogenase gene from *Escherichia coli* to *Methylophilus methylotrophus*
Enzyme for degrading cell walls for protein concentrate production	*Rhizoctonia solani* 1,3-β-D-glucanase for degrading yeast cell walls
Extracellular production of proteins	Excretion of protein into medium by *Bacillus brevis*
Improved harvesting methods	Agglomeration, electrocoagulation
Automation of production	Computer control of Pekilo process

UNIT – III

MICROBIAL INOCULANTS

Biofertilizers

Biofertilizers are defined as preparations containing living cells or latent cells of efficient strains of microorganisms that help crop plants' uptake of nutrients by their interactions in the rhizosphere when applied through seed or soil. They accelerate certain microbial processes in the soil which augment the extent of availability of nutrients in a form easily assimilated by plants.

Very often microorganisms are not as efficient in natural surroundings as one would expect them to be and therefore artificially multiplied cultures of efficient selected microorganisms play a vital role in accelerating the microbial processes in soil. Use of biofertilizers is one of the important components of integrated nutrient management, as they are cost effective and renewable source of plant nutrients to supplement the chemical fertilizers for sustainable agriculture. Several microorganisms and their association with crop plants are being exploited in the production of biofertilizers. They can be grouped in different ways based on their nature and function.

S.No.	Groups	Examples
N2 fixing Biofertilizers		
1.	Free-living	*Azotobacter, Beijerinkia, Clostridium, Klebsiella, Anabaena, Nostoc,*
2.	Symbiotic	*Rhizobium, Frankia, Anabaena azollae*
3.	Associative Symbiotic	*Azospirillum*
P Solubilizing Biofertilizers		
1.	Bacteria	*Bacillus megaterium* var. *phosphaticum, Bacillus subtilis* *Bacillus circulans, Pseudomonas striata*
2.	Fungi	*Penicillium sp, Aspergillus awamori*
P Mobilizing Biofertilizers		
1.	Arbuscular mycorrhiza	*Glomus sp.,Gigaspora sp.,Acaulospora sp., Scutellospora sp.* & *Sclerocystis sp.*
2.	Ectomycorrhiza	*Laccaria sp., Pisolithus sp., Boletus sp., Amanita sp.*
3.	Ericoid mycorrhizae	*Pezizella ericae*
4.	Orchid mycorrhiza	*Rhizoctonia solani*
Biofertilizers for Micro nutrients		
1.	Silicate and Zinc solubilizers	*Bacillus* sp.
Plant Growth Promoting Rhizobacteria		
1.	Pseudomonas	*Pseudomonas fluorescens*

Different types of biofertilizers

Rhizobium

Rhizobium is a soil habitat bacterium, which can able to colonize the legume roots and fixes the atmospheric nitrogen symbiotically. The morphology and physiology of Rhizobium will vary from free-living condition to the bacteroid of nodules. They are the most efficient biofertilizer as per the quantity of nitrogen fixed concerned. They have seven genera and highly specific to form nodule in legumes, referred as cross inoculation group. *Rhizobium* inoculant was first made in USA and commercialized by private enterprise in 1930s and the strange situation at that time has been chronicled by Fred (1932).

Initially, due to absence of efficient bradyrhizobial strains in soil, soybean inoculation at that time resulted in bumper crops but incessant inoculation during the last four decades by US farmers has resulted in the build up of a plethora of inefficient strains in soil whose replacement by efficient strains of bradyrhizobia has become an insurmountable problem.

Azotobacter

Of the several species of *Azotobacter*, *A. chroococcum* happens to be the dominant inhabitant in arable soils capable of fixing N2 (2-15 mg N2 fixed /g of carbon source) in culture media. The bacterium produces abundant slime which helps in soil aggregation. The numbers of *A. chroococcum* in Indian soils rarely exceeds 105/g soil due to lack of organic matter and the presence of antagonistic microorganisms in soil.

Azospirillum

Azospirillum lipoferum and *A. brasilense* (*Spirillum lipoferum* in earlier literature) are primary inhabitants of soil, the rhizosphere and intercellular spaces of root cortex of graminaceous plants. They perform the associative symbiotic relation with the graminaceous plants. The bacteria of Genus *Azospirillum* are N2 fixing organisms isolated from the root and above ground parts of a variety of crop plants. They are Gram negative, *Vibrio* or *Spirillum* having abundant accumulation of polybetahydroxybutyrate (70 %) in cytoplasm.

Five species of *Azospirillum* have been described to date *A. brasilense*, *A.lipoferum*, *A.amazonense*, *A.halopraeferens* and *A.irakense*.

The organism proliferates under both anaerobic and aerobic conditions but it is preferentially micro-aerophilic in the presence or absence of combined nitrogen in the medium. Apart from nitrogen fixation, growth promoting substance production (IAA), disease resistance and drought tolerance are some of the additional benefits due to *Azospirillum* inoculation.

Cyanobacteria

Both free-living as well as symbiotic cyanobacteria (blue green algae) have been harnessed in rice cultivation in India. A composite culture of BGA having heterocystous *Nostoc*, *Anabaena*, *Aulosira etc*. is given as primary inoculum in trays, polythene lined pots and later mass multiplied in the field for application as soil based flakes to the rice growing field at the rate of 10 kg/ha. The final product is not free from extraneous contaminants and not very often monitored for checking the presence of desiredalgal flora.

Once so much publicized as a biofertilizer for the rice crop, it has not presently attracted the attention of rice growers all over India except pockets in the Southern States, notably Tamil Nadu. The benefits due to algalization could be to the extent of 20-30 kg N/ha under ideal conditions but the labour oriented methodology for the preparation of BGA biofertilizer is in itself a limitation. Quality control measures are not usually followed except perhaps for random checking for the presence of desired species qualitatively.

Azolla

Azolla is a free-floating water fern that floats in water and fixes atmospheric nitrogen in association with nitrogen fixing blue green alga *Anabaena azollae*. *Azolla* fronds consist of sporophyte with a floating rhizome and small overlapping bi-lobed leaves and roots. Rice growing areas in South East Asia and other third World countries have recently been evincing increased interest in the use of the symbiotic N2 fixing water fern *Azolla* either as an alternate nitrogen sources or as a supplement to commercial nitrogen fertilizers. *Azolla* is used as biofertilizer for wetland rice and it is known to contribute 40-60 kg N/ha per rice crop.

Phosphate solubilizing microorganisms(PSM)

Several soil bacteria and fungi, notably species of *Pseudomonas, Bacillus, Penicillium, Aspergillus etc*. secrete organic acids and lower the pH in their vicinity to bring about dissolution of bound phosphates in soil. Increased yields of wheat and potato were demonstrated due to inoculation of peat based cultures of *Bacillus polymyxa* and *Pseudomonas striata*. Currently, phosphate solubilizers are manufactured by agricultural universities and some private enterprises and sold to farmers through governmental agencies. These appear to be no check on either the quality of the inoculants marketed in India or the establishment of the desired organisms in the rhizosphere.

AM fungi

The transfer of nutrients mainly phosphorus and also zinc and sulphur from the soil *milleu* to the cells of the root cortex is mediated by intracellular obligate fungal endosymbionts of the genera *Glomus, Gigaspora, Acaulospora, Sclerocysts* and *Endogone* which possess vesicles for storage of nutrients and arbuscles for funneling these nutrients into the root system. By far, the commonest genus appears to be *Glomus*, which has several species distributed in soil. Availability for pure cultures of AM (Arbuscular Mycorrhiza) fungi is an impediment in large scale production despite the fact that beneficial effects of AM fungal inoculation to plants have been repeatedly shown under experimental conditions in the laboratory especially in conjunction with other nitrogen fixers.

Silicate solubilizing bacteria (SSB)

Microorganisms are capable of degrading silicates and aluminum silicates. During the metabolism of microbes several organic acids are produced and these have a dual role in silicate weathering. They supply H+ ions to the medium and promote hydrolysis and the organic acids like citric, oxalic acid, Keto acids and hydroxy carbolic acids which from complexes with cations, promote their removal and retention in the medium in a dissolved state.

The studies conducted with a Bacillus sp. isolated from the soil of granite crusher yard showed that the bacterium is capable of dissolving several silicate minerals under *in vitro* condition. The examination of anthrpogenic materials like cement, agro inputs like super phosphate and rock phosphate exhibited silicate solubilizing bacteria to a varying degree. The bacterial isolates made from different locations had varying degree of silicate solubilizing potential.

Soil inoculation studies with selected isolate with red soil, clay soil, sand and hilly soil showed that the organisms multiplied in all types of soil and released more of silica and the available silica increased in soil and water. Rice responded well to application of organic sliceous residue like rice straw, rice husk and black ash @ 5 t/ha. Combining SSB with these residues further resulted in increased plant growth and grain yield. This enhancement is due to increased dissolution of silica and nutrients from the soil.

Plant Growth Promoting Rhizobacteria (PGPR)

The group of bacteria that colonize roots or rhizosphere soil and beneficial to crops are referred to as plant growth promoting rhizobacteria (PGPR).

The PGPR inoculants currently commercialized that seem to promote growth through at least one mechanism; suppression of plant disease (termed Bioprotectants), improved nutrient acquisition (termed Biofertilizers), or phytohormone production (termed Biostimulants). Species of *Pseudomonas* and *Bacillus* can produce as yet not well characterized phytohormones or growth regulators that cause crops to have greater amounts of fine roots which have the effect of increasing the absorptive surface of plant roots for uptake of water and nutrients. These PGPR are referred to as Biostimulants and the phytohormones they produce include indole-acetic acid, cytokinins, gibberellins and inhibitors of ethylene production.

Recent advances in molecular techniques also are encouraging in that tools are becoming available to determine the mechanism by which crop performance is improved using PGPR and track survival and activity of PGPR organisms in soil and roots. The science of PGPR is at the stage where genetically modified PGPR can be produced. PGPR with antibiotic, phytohormone and siderophore production can be made.

Despite of promising results, biofertilizers has not got widespread application in agriculture mainly because of the variable response of plant species or genotypes to inoculation depending on the bacterial strain used. Differential rhizosphere effect of crops in harbouring a target strain or even the modulation of the bacterial nitrogen fixing and phosphate solubilizing capacity by specific root exudates may account for the observed differences.

On the other hand, good competitive ability and high saprophytic competence are the major factors determining the success of a bacterial strain as an inoculant. Studies to know the synergistic activities and persistence of specific microbial populations in complex environments, such as the rhizosphere, should be addressed in order to obtain efficient inoculants. In this regards, research efforts are made at Agricultural College and Research Institute, Madurai to obtain appropriate formulations of microbial inoculants incorporating nitrogen fixing, phosphate- and silicate- solubilizing bacteria and plant growth promoting rhizobacteria which will help in promoting the use of such beneficial bacteria in sustainable agriculture.

Liquid Biofertilizers

Biofertilizers are such as *Rhizobium, Azospirillum* and Phosphobacteria provide nitrogen and phosphorous nutrients to crop plants through nitrogen fixation and phosphorous solubilization processes. These Biofertilizers could be effectively utilized for rice, pulses, millets, cotton, sugarcane, vegetable and other horticulture crops. Biofertilizers is one of the prime input in organic farming not only enhances the crop growth and yield but also improves the soil health and sustain soil fertility. At present, Biofertilizers are supplied to the farmers as carrier based inoculants. As an alternative, liquid formulation technology has been developed in the Department of Agricultural Microbiology, TNAU, Coimbatore which has more advantages than the carrier inoculants.

Benefits

The advantages of Liquid Bio-fertilizer over conventional carrier based Bio-fertilizers are listed below:

- Longer shelf life -12-24 months.
- No contamination.
- No loss of properties due to storage upto 45° c.
- Greater potentials to fight with native population.
- High populations can be maintained more than 109 cells/ml upto 12 months to 24 months.
- Easy identification by typical fermented smell.
- Cost saving on carrier material, pulverization, neutralization, sterilization, packing and transport.

- Quality control protocols are easy and quick.
- Better survival on seeds and soil.
- No need of running Bio-fertilizer production units through out the year.
- Very much easy to use by the farmer.
- Dosages is 10 time less than carrier based powder Bio-fertilizers.
- High commercial revenues.
- High export potential.
- Very high enzymatic activity since contamination is nil.

Characteritistics of different liquid Bio-fertilizers

Rhizobium

This belongs to bacterial group and the classical example is symbiotic nitrogen fixation. The bacteria infect the legume root and form root nodules within which they reduce molecular nitrogen to ammonia which is reality utilized by the plant to produce valuable proteins, vitamins and other nitrogen containing compounds. The site of symbiosis is within the root nodules. It has been estimated that 40-250 kg N / ha / year is fixed by different legume crops by the microbial activities of *Rhizobium*. The percentage of nodules occupied, nodules dry weight, plant dry weight and the grain yield per plant the multistrain inoculant was highly promising Table-2 shows the N fixation rates.

Quantity of biological N fixed by Liqiud *Rhizobium* in different crops

Host Group	***Rhizobium* Species**	**Crops**	**N fix kg/ha**
Pea group	*Rhizobium leguminosarum*	Green pea, Lentil	62- 132
Soybean group	*R.japonicum*	Soybean	57- 105
Lupini Group	*R. lupine orinthopus*	Lupinus	70- 90
Alfafa grp.Group	*R.mellilotiMedicago Trigonella*	Melilotus	100- 150
Beans group	*R. phaseoli*	Phaseoli	80- 110
Clover group	*R. trifoli*	Trifolium	130
Cowpea group	*R. species*	Moong, Redgram, Cowpea, Groundnut	57- 105
Cicer group	*R. species*	Bengal gram	75- 117

Physical features of liquid Rhizobium

- Dull white in colour
- No bad smell
- No foam formation, pH 6.8-7.5

Azospirillum

It belongs to bacteria and is known to fix the considerable quantity of nitrogen in the range of 20- 40 kg N/ha in the rhizosphere in non- non-leguminous plants such as cereals, millets, Oilseeds, cotton etc. The efficiency of *Azospirillium* as a Bio-Fertilizer has increased because of its ability of inducing abundant roots in several pants like rice, millets and oilseeds even in upland conditions. Considerable quantity of nitrogen fertilizer up to 25-30 % can be saved by the use of *Azospirillum* inoculant. The genus *Azospirillum* has three species viz., *A. lipoferum*, *A. brasilense* and *A. amazonense*. These species have been commercially exploited for the use as nitrogen supplying Bio-Fertilizers.

One of the characteristics of *Azospirillum* is its ability to reduce nitrate and denitrify. Both *A. lipoferum*,and *A. brasilense* may comprise of strains which can actively or weakly denitrify or reduce nitrate to nitrite and therefore, for inoculation preparation, it is necessary to select strains which do not possess these characteristics. *Azospirllium lipoferum* present in the roots of some of tropical forage grasses uch as Digitaria, Panicum, Brachiaria, Maize, Sorghum, Wheat and Rye.

Physical features of liquid *Azospirillum*

- The colour of the liquid may be blue or dull white.
- Bad odours confirms improper liquid formulation and may be concluded as mere broth.
- Production of yellow gummy colour materials comfirms the quality product.
- Acidic pH always confirms that there is no Azospirillum bacteria in the liquid.

N_2 fixing capacity of *Azospirillum* in the roots of several plants and the amount of N_2 fixed by them

Plant	Mg N2 fixed /g of substrate
Oryza sativa (Paddy)	28
Sorghum bicolour (Sorghum)	20
Zea mays (Maize)	20
Panicum sp.	24
Cynodon dactylon	36
Setaria sp	12
Amaranthus spinosa	16

Production of growth hormones

Azospirillum cultures synthesize considerable amount of biologically active substances like vitamins, nicotinic acid, indole acetic acids giberllins. All these hormones/chemicals helps the plants in better germination, early emergence, better root development.

Role of Liquid *Azospirillum* under field conditions

- Stimulates growth and imparts green colour which is a characteristic of a healthy plant.
- Aids utilization of potash, phosphorous and other nutrients.
- Encourage plumpness and succulence of fruits and increase protein percentage.

Sign of non functioning of *Azospirillum* in the field

- No growth promotion activity
- Yellowish green colour of leaves, which indicates no fixation of Nitrogen

Azotobacter

It is the important and well known free living nitrogen fixing aerobic bacterium. It is used as a Bio-Fertilizer for all non leguminous plants especially rice, cotton, vegetables etc. *Azotobacter* cells are not present on the rhizosplane but are abundant in the rhizosphere region. The lack of organic matter in the soil is a limiting factor for the proliferation of *Azotobaceter* in the soil.

Field experiments were conducted in 1992, 1993 and 1994 during the pre-kharif wet seasons to find out the influence on rice grain yield by the combined use of N- fixing organisms and inorganic nitrogen fertilizer which recorded increase in was yield.

Physical features of liquid *Azotobacter*

The pigmentation that is produced by Azotobacter in aged culture is melanin which is due to oxidation of tyrosine by tyrosinase an enzyme which has copper. The colour can be noted in liquid forms. Some of the pigmentation are described below-

- *A. chroococcum*: Produces brown-black pigmentation in liquid inoculum.
- *A. beijerinchii*: Produces yellow- light brown pigementation in liquid inoculum
- *A. vinelandii*: Produces green fluorescent pigmentation in liquid inoculum.
- *A. paspali*: Produces green fluorescent pigmentation in liquid inoculum.
- *A. macrocytogenes*: Produces, pink pigmentation in liquid inoculum.
- *A. insignis*: Produces less, gum less, grayish-blue pigmentation in liquid inoculum.
- *A. agilies*: Produces green-fluorescent pigmentation in liquid inoculum.

Role of liquid *Azotobacter* in tissue culture

The study was conducted by Dr. Senthil et al (2004) on sugarcane variety CO 86032 in Tissue culture Laboratories of Rajashree Sugars and Chemicals Ltd, Varadaraj nagar, Theni, Tamilnadu. The liquid bioinoculants were provided by Dr. Krishnan Chandra, Regional Director, RCOF, Bangalore to evaluate their growth promoting effects on sugarcane micropropagation. He recorded Biometric observations like Plant height, leaf length, width, root length, no of roots. Chemical parameters –Protein, Carbohydrates, N, P,K total biomass and concluded as follows:

- The performance of *Azotobacter* liquid inoculant was c
- omparatively better than all the treatments in 10 % MS medium followed Azospirillum.
- The performance of *Azotobacter* liquid inoculant was comparatively better than all the treatments followed by Azosopirillum for the growth of the polybag sugarcane seedlings.

Role of liquid Azotobacter as a Bio-control agent

Azotobacter have been found to produce some antifungal substance which inhibits the growth of some soil fungi like *Aspergillus, Fusarium, Curvularia, Alternaria, Helminthosporium, Fusarium* etc.

Acetobaceter

This is a sacharophillic bacteria and associate with sugarcane, sweet potato and sweet sorghum plants and fixes 30 kgs/ N/ ha year. Mainly this bacterium is commercialized for sugarcane crop. It is known to increase yield by 10-20 t/ acre and sugar content by about 10-15 percent.

Effect of liquid *Acetobacter diazotrophicus* on sugarcane

In South India use of Azospirillum and Phospho-bacterium on the cash crop sugarcane is a regular practice for the past few years with a saving of nearly 20 % of chemical nitrogen and phosphate applications. Now, it has been reported that a bacteria *Acetobacter diazotrophicus* which is present in the sugarcane stem, leaves, soils have a capacity to fix up to 300 kgs of nitrogen. This bacteria first reported in brazil where the farmers cultivate sugarcane in very poor sub-soil fertilized with Phosphate, Potassium and micro elements alone, could produce yield for three consecutive harvests, without any nitrogen fertilizer. They have recorded yield 182-244 tones per ha. This leads to the assumption that active nitrogen fixing bacteria has associated within the plant.

Do's and Don't for Entrepreneurs, Dealers and farmers

Do	Don't
Keep Bio-fertilizers bottles away from direct heat and sunlight. Store it in cool and dry place.	Don't store Bio-fertilizers bottles under heat and sunlight
Sell only Bio-fertilizers bottles which contain batch number, the name of the crop on which it has to be used, the date of manufacture and expiry period.	Don't sell Bio-fertilizers bottles after their expiry period is over.
If the expiry period is over, then discard it as it is not effective.	Don't prick holes into the bottles or puncture them to pour the content

Keep Bio-fertilizers bottles away from fertilizer or pesticide containers and they should not be mixed directly.	Do not mix the Bio-fertilizers with fungicides, insecticides, herbicides, herbicides and chemical fertilizers.

Liquid Bio-fertlizer application methodology

There are three ways of using Liquid Bio-fertilizers

1. Seed treatment
2. Root dipping
3. Soil application

Seed Treatment

Seed Treatment is a most common method adopted for all types of inoculants. The seed treatment is effective and economic. For small quantity of seeds (up to 5 kgs quantity) the coating can done in a plastic bag. For this purpose, a plastic bag having size (21" x 10") or big size can be used. The bag should be filled with 2 kg or more of seeds. The bag should be closed in such a way to trap the airs as much as possible. The bag should be squeezed for 2 minutes or more until all the seed are uniformly wetted. Then bag is opened, inflated again and shaked gently. Stop shaking after each seeds gets a uniform layer of culture coating. The bag is opened and the seed is dried under the shade for 20-30 minutes. For large amount of seeds coating can be done in a bucket and inoculant can be mixed directly with hand. Seed Treatment with *Rhizobium*, *Azotobacter, Azospirillum*, along with PSM can be done.

The seed treatment can be done with any of two or more bacteria. There is no side (antagonistic) effect. The important things that has to be kept in mind are that the seeds must be coated first with *Rhizobium, Azotobacter* or *Azospirillum*. When each seed get a layer of above bacteria then PSM inoculant has to be coated as outer layer. This method will provide maximum number of each bacteria required for better results. Treatments of seed with any two bacteria will not provide maximum number of bacteria on individual seed.

Root dipping

For application of *Azospirillum*/ /PSM on paddy transplating/ vegetable crops this method is used. The required quantity of *Azospirillum*/ /PSM has to be mixed with 5-10 litres of water at one corner of the field and the roots of seedlings has to be dipped for a minimum of half-an-hour before transplantation.

Soil application

Use 200ml of PSM per acre. Mix PSM with 400 to 600 kgs of Cow dung FYM along with ½ bag of rock phosphate if available. The mixture of PSM, cow dung and rock phosphate have to be kept under any tree or under shade for over night and maintain 50% moisture. Use the mixture as soil application in rows or during leveling of soil.

Dosage of liquid Bio-fertilizers in different crops

Recommended Liquid Bio-fertilizers and its application method, quantity to be used for different crops are as follows:

Crop	Recommended Bio-fertilizer	Application method	Quantity to be used
Field crops Pulses - Chickpea, pea, Groundnut, soybean, beans, Lentil, lucern, Berseem, Green gram, Black gram, Cowpea and pigeon pea	*Rhizobium*	Seed treatment	200ml/acre
Cereals - Wheat, oat, barley	*Azotobacter/Azospirillum*	Seed treatment	200ml/acre
Rice	*Azospirillum*	Seed treatment	200ml/acre
Oil seeds - Mustard, seasum, Linseeds, Sunflower, castor	*Azotobacter*	Seed treatment	200ml/acre
Millets - Pearl millets, Finger millets, kodo millet	*Azotobacter*	Seed treatment	200ml/acre
Maize and Sorghum	*Azospirillum*	Seed treatment	200ml/acre
Forage crops and Grasses Bermuda grass, Sudan grass, Napier Grass , ParaGrass, Star Grass etc.	*Azotobacter*	Seed treatment	200ml/acre
Other Misc. Plantation Crops Tobacco	*Azotobacter*	Seedling treatment	500ml/acre
Tea, Coffee	*Azotobacter*	Soil treatment	400ml/acre

Rubber, Coconuts	*Azotobacter*	Soil treatment	2-3 ml/plant
Agro-ForestRY/Fruit Plants All fruit/agro-forestry (herb,shrubs, annuals and perennial) plants for fuel wood fodder, fruits,gum,spice,leaves,flowers,nuts and seeds puppose	*Azotobacter*	Soil treatment	2-3 ml/plant at nursery
Leguminous plants/ trees	*Rhizobium*	Soil treatment	1-2 ml/plant

Note:

Doses recommended when count of inoculum is 1 x 10^8 cells/ml then doses will be ten times more besides above said Nitrogen fixers, Phosphate solubilizers and potash mobilizers at the rate of 200 ml/ acre could be applied for all crops.

Equipments required for Biofertilizer production

In biofertilizer production industry, equipments are the major infrastructure, which involves 70 percent of capital investment. Any compromise on the usage of the following mentioned equipments may finally decline in the quality of biofertilizer.After studying the principle behind the usage of all instruments, some of the instruments can be replaced with a culture room fitted with a U.V.Lamp. Autoclaves, Hot Air Oven, Incubators and sealing machines are indigenously made with proper technical specifications. The correct use of equipments will give uninterrupted introduction with quality inoculum.

Essential equipments

Autoclave

It is an apparatus in which materials are sterilized by air free saturated steam (under pressure) at a temperature above 100OC. If the steam pressure inside the autoclave is increased to 15 psi, the temperature will rise to 121°C. this is sufficient to destroy all vegetative cells. Normally all growth medium are sterilized in the autoclave.

Laminar air flow chamber

Laminar air flow chamber provides a uniform flow of filtered air. This continuous flow of air will prevent settling of particles in the work area.Air borne contamination is avoided in this chamber. Culture transfers and inoculation can be done here.

BOD incubators

Incubators providing controlled conditions (light, temperature, humidity, etc.) required for the growth and development of microorganisms. Multiplication of starter culture can be done in this instrument.

Rotary shaker

It is used for agitating culture flasks by circular motion under variable speed control. Shaking provides aeration for growth of cultures. Shakers holding upto 20-50 flasks are generally used. The capacity of the shaker may be increased if it is a double- decker type.

Hot air oven

Hot air oven is meant for sterilizing all glassware materials. Dry heat is used in this apparatus to sterilize the materials. Normally 180OC is used for two hours for sterilizing glasswares.

pH meter

An instrument for measuring pH of the solution using a 0-14 scale in which seven represents neutral points, less than seven is acidity (excess of H‘ over OH-) and more than seven is alkality (excess of OH- over H‘) useful in adjusting the pH of the growth medium.

Refrigerator

This equipment is used preserving all mother cultures used for biofertilizer production. The mother culture is periodically sub-cultured and stored in the refrigerator for long- term usage.

Fermentor

A fermentor is the equipment, which provides the proper environment for the growth of a desired organism. It is generally a large vessel in which, the organism may be kept at the required temperature, pH , dissolved oxygen concentration and substrate concentration. Different models of fermentors are available depending upon the necessity.

A simple version model contains steam generator, sterilization process devices and agitator. A sophisticated fermentor contains pH regulator, oxygen level regulator, anti-foam device, temperature controller, etc.

Mass production of Bacterial Biofertilizer

Biofertilizers are carrier based preparations containing efficient strain of nitrogen fixing or phosphate solubilizing microorganisms. Biofertilizers are formulated usually as carrier based inoculants. The organic carrier materials are more effective for the preparation of bacterial inoculants. The solid inoculants carry more number of bacterial cells and support the survival of cells for longer periods of time.

- The mass production of carrier based bacterial biofertilizers involves three stages.
- Culturing of microorganisms
- Processing of carrier material
- Mixing the carrier and the broth culture and packing

Culturing of Microorganisms

Although many bacteria can be used beneficially as a biofertilizer the technique of mass production is standardizedfor *Rhizobium*, *Azospirillum*, *Azotobacter* and phosphobacteria.

The media used for mass culturing are as follows:

Rhizobium : Yeast extract mannitol broth.

Growth on Congo red yeast extract mannitol agar medium

Mannitol	-	10.0 g
K2 HPO4	-	0.5 g
Mg So4 7H2 O	-	0.2 g
NaCl	-	0.1 g
Yeast extract	-	0.5 g
Agar		20.0 g
Distilled water		1000.0 ml

Add 10 ml of Congo red stock solution (dissolve 250 mg of Congo red in 100ml water) to 1 liter after adjusting the PH to 6.8 and before adding agar.

Rhizobium forms white, translucent, glistening, elevated and comparatively small colonies on this medium. Moreover, *Rhizobium* colonies do not take up the colour of congo red dye added in the medium. Those colonies which readily take up the congo red stain are not rhizobia but presumably *Agrobacterium*, a soil bacterium closely related to *Rhizobium.*

Azospirillum : Dobereiner's malic acid broth with NH4Cl (1g per liter)

Composition of the N-free semisolid malic acid medium

Malic acid	-	5.0g
Potassium hydroxide	-	4.0g
Dipotassium hydrogen-orthophosphate	-	0.5g
Magnesium sulphate	-	0.2g
Sodium chloride	-	0.1g
Calcium chloride	-	0.2g
Fe-EDTA (1.64% w/v aqueous)	-	4.0 ml
Trace element solution	-	2.0 ml
BTB (0.5% alcoholic solution)	-	2.0 ml
Agar	-	1.75 g
Distilled water	-	1000 ml
pH	-	6.8
Trace element solution		
Sodium molybdate	-	200 mg
Manganous sulphate	-	235 mg
Boric acid	-	280 mg
Copper sulphate	-	8 mg
Zinc sulphate	-	24 mg
Distilled water	-	200 ml

Waksman medium No.77 (N-free Mannitol Agar Medium for *Azotobacter*)

Mannitol	: 10.0 g
Ca CO3	: 5.0 g
K2HPO4	: 0.5 g
Mg SO4.7H2O	: 0.2 g
NaCl	: 0.2 g
Ferric chloride	: Trace
MnSO4.4H2O	: Trace
N-free washed Agar	: 15.0 g
pH	: 7.0
Distilled Water	: 1000 ml

Phosphobacteria : Pikovskaya's Broth

Glucose	10.0 g
Ca3(PO4)2	5.0 g
(NH4)2SO4	0.5 g
KCl	0.2 g
MgSO4. 7H2O	0.1 g
MnSO4	Trace
FeSO4	Trace
Yeast Extract	0.5 g
Distilled Water	1000 ml

The broth is prepared in flasks and inoculum from mother culture is transferred to flasks. The culture is grown under shaking conditions at 30±2°C as submerged culture. The culture is incubated until maximum cell population of 1010 to 1011 cfu/ml is produced. Under optimum conditions this population level could be attained with in 4 to 5 days for *Rhizobium*; 5 to 7 days for *Azospirillum*; 2 to 3 days for phosphobacteria and 6-7 days for *Azotobacter*. The culture obtained in the flask is called **starter culture**. For large scale production of inoculant, inoculum from starter culture is transferred to large flasks/seed tank fermentor and grown until required level of cell count is reached.

Inoculum preparation

- Prepare appropriate media for specific to the bacterial inoculant in 250 ml, 500 ml, 3 litre and 5 litre conical flasks and sterilize.
- The media in 250 ml flask is inoculated with efficient bacterial strain under aseptic condition
- Keep the flask under room temperature in rotary shaker (200 rpm) for 5- 7 days.
- Observe the flask for growth of the culture and estimate the population, which serves as the starter culture.
- Using the starter culture (at log phase) inoculate the larger flasks (500 ml, 3 litre and 5 litre) containing the media, after obtaining growth in each flask.
- The above media is prepared in large quantities in fermentor, sterilized well, cooled and kept it ready.
- The media in the fermentor is inoculated with the log phase culture grown in 5 litre flask. Usually 1 -2 % inoculum is sufficient, however inoculation is done up to 5% depending on the growth of the culture in the larger flasks.
- The cells are grown in fermentor by providing aeration (passing sterile air through compressor and sterilizing agents like glass wool, cotton wool, acid etc.) and given continuous stirring.
- The broth is checked for the population of inoculated organism and contamination if any at the growth period.
- The cells are harvested with the population load of 109 cells ml-1 after incubation period.
- There should not be any fungal or any other bacterial contamination at 10-6 dilution level

- It is not advisable to store the broth after fermentation for periods longer than 24 hours. Even at 4o C number of viable cells begins to decrease.

Processing of carrier material

The use of ideal carrier material is necessary in the production of good quality biofertilizer. Peat soil, lignite, vermiculite, charcoal, press mud, farmyard manure and soil mixture can be used as carrier materials. The neutralized peat soil/lignite are found to be better carrier materials for biofertilizer production The following points are to be considered in the selection of ideal carrier material.

- Cheaper in cost
- Should be locally available
- High organic matter content
- No toxic chemicals
- Water holding capacity of more than 50%
- Easy to process, friability and vulnerability.

Preparation of carrier material

- The carrier material (peat or lignite) is powdered to a fine powder so as to pass through 212 micron IS sieve.
- The pH of the carrier material is neutralized with the help of calcium carbonate (1:10 ratio) , since the peat soil / lignite are acidic in nature (pH of 4 - 5)
- The neutralized carrier material is sterilized in an autoclave to eliminate the contaminants.

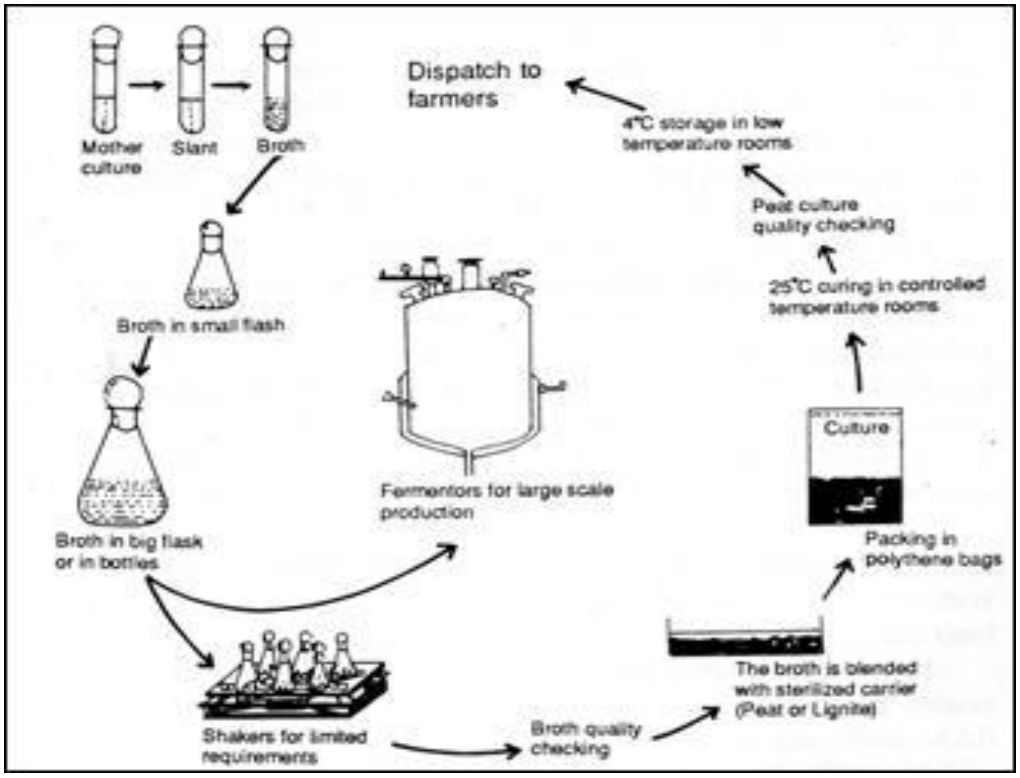
Mixing the carrier and the broth culture and packing

Inoculant packets are prepared by mixing the broth culture obtained from fermentor with sterile carrier material as described below:

Preparation of Inoculants packet

- The neutralized, sterilized carrier material is spread in a clean, dry, sterile metallic or plastic tray.
- The bacterial culture drawn from the fermentor is added to the sterilized carrier and mixed well by manual (by wearing sterile gloves) or by mechanical mixer. The culture suspension is to be added to a level of 40 – 50% water holding capacity depending upon the population.

- The inoculant packet of 200 g quantities in polythene bags, sealed with electric sealer and allowed for curing for 2 -3 days at room temperature (curing can be done by spreading the inoculant on a clean floor/polythene sheet/ by keeping in open shallow tubs/ trays with polythene covering for 2 -3 days at room temperature before packaging).



schematic representation of mass production of bacterial biofertilizers

Specification of the polythene bags

- The polythene bags should be of low density grade.
- The thickness of the bag should be around 50 – 75 micron.
- Each packet should be marked with the name of the manufacturer, name of the product, strain number, the crop to which recommended, method of inoculation, date of manufacture, batch number, date of expiry, price, full address of the manufacturer and storage instructions etc.,

Storage of biofertilizer packet

- The packet should be stored in a cool place away from the heat or direct sunlight.
- The packets may be stored at room temperature or in cold storage conditions in lots in plastic crates or polythene / gunny bags.
- The population of inoculant in the carrier inoculant packet may be determined at 15 days interval. There should be more than 109 cells / g of inoculant at the time of preparation and107 cells/ g on dry weight basis before expiry date.

Mass production of Mycorrhizal biofertilizer

he commercial utilization of mycorrhizal fungi has become difficult because of the obligate symbiotic nature and difficulty in culturing on laboratory media. Production of AM inoculum has evolved from the original use of infested field soils to the current practice of using pot culture inoculum derived from the surface disinfected spores of single AM fungus on a host plant grown in sterilized culture medium. Several researches in different parts of the world resulted in different methods of production of AM fungal inoculum as soil based culture as well as carrier based inoculum. Root organ culture and nutrient film technique provide scope for the production of soil less culture.

As a carrier based inoculum, pot culture is widely adopted method for production. The AM inoculum was prepared by using sterilized soil and wide array of host crops were used as host. The sterilization process is a cumbersome one and scientists started using inert materials for production of AM fungi. The researchers tried use of perlite, montmorillonite clay etc., In TNAU vermiculite was tried as substrate for the replacement of soil sterilization, which resulted in the best method of inoculum production.

METHOD OF PRODUCTION

- A trench (1m x 1m x 0.3m) is formed and lined with black polythene sheet to be used as a plant growth tub.
- Mixed 50 kg of vermiculite and 5 kg of sterilized soil and packed in the trench up to a height of 20 cm
- Spread 1 kg of AM inoculum (mother culture) 2-5 cm below the surface of vermiculite

- Maize seeds surface sterilized with 5% sodium hypochlorite for 2 minutes are sown
- Applied 2 g urea, 2 g super phosphate and 1 g muriate of potash for each trench at the time of sowing seeds. Further 10 g of urea is applied twice on 30 and 45 days after sowing for each trench
- Quality test on AM colonization in root samples is carried out on 30th and 45th day
- Stock plants are grown for 60 days (8 weeks). The inoculum is obtained by cutting all the roots of stock plants. The inoculum produced consists of a mixture of vermiculite, spores, pieces of hyphae and infected root pieces.
- Thus within 60 days 55 kg of AM inoculum could be produced from 1 sq meter area. This inoculum will be sufficient to treat 550 m2 nursery area having 11,000 seedlings.

AM fungi

Nursery application: 100 g bulk inoculum is sufficient for one metre square. The inoculum should be applied at 2-3 cm below the soil at the time of sowing. The seeds/cutting should be sown/planted above the VAM inoculum to cause infection.

For polythene bag raised crops: 5 to 10 g bulk inoculum is sufficient for each packet. Mix 10 kg of inoculum with 1000 kg of sand potting mixture and pack the potting mixture in polythene bag before sowing.

For out –planting:

Twenty grams of VAM inoculum is required per seedling. Apply inoculum at the time of planting.

For existing trees: Two hundred gram of VAM inoculum is required for inoculating one tree. Apply inoculum near the root surface at the time of fertilizer application.

Mass production and field application of cyanobacteria

Blue green algal inoculation with composite cultures was found to be more effective than single culture inoculation. A technology for mass scale production of composite culture of blue green algae under rice field condition was developed at TNAU and the soil based BGA inoculum could survive for more than 2 years.

At many sites where algal inoculation was used for three to four consecutive cropping seasons, the inoculated algae establish well and the effect persisted over subsequent rice crop. Technologies for utilizing nitrogen fixing organisms in low land rice were the beneficial role of blue green algal inoculation in rice soils of Tamil Nadu. The blue green algal inoculum may be produced by several methods viz., in tubs, galvanized trays, small pits and also in field conditions. However the large-scale production is advisable under field condition which is easily adopted by farmers.

I. Multiplication in trays

- Big metallic trays (6'x 3'x 6"lbh) can be used for small scale production
- Take 10 kg of paddy field soil, dry powder well and spread
- Fill water to a height of 3"
- Add 250 g of dried algal flakes (soil based) as inoculum
- Add 150 g of super phosphate and 30 g of lime and mix well with the soil
- Sprinkle 25 g carbofuran to control the insects
- Maintain water level in trays
- After 10 to 15 days, the blooms of BGA will start floating on the water sources
- At this stage stop watering and drain. Let the soil to dry completely
- Collect the dry soil based inoculum as flakes
- Store in a dry place. By this method 5 to 7 kg of soil based inoculum can be obtained.

II. Multiplication under field condition

Materials

- Rice field
- Super phosphate
- Carbofuran
- Composite BGA starter culture

Procedure

Select an area of 40 m2 (20m x 2m) near a water source which is directly exposed to sunlight.

Make a bund all around the plot to a height of 15 cm and give it a coating with mud to prevent loss of water due to percolation.

- Plot is well prepared and levelled uniformly and water is allowed to a depth of 5-7.5 cm and left to settle for 12 hrs.
- Apply 2 kg of super phosphate and 200 g lime to each plot uniformly over the area.
- The soil based composite starter culture of BGA containing 8-10 species @ 5 kg / plot is powdered well and broadcasted.
- Carbofuran @ 200 g is also applied to control soil insects occurring in BGA.
- Water is let in at periodic intervals so that the height of water level is always maintained at 5 cm.
- After 15 days of inoculation, the plots are allowed to dry up in the sun and the algal flakes are collected and stored.

Observations

The floating algal flasks are green or blue green in colour. From each harvest, 30 to 40 kg of dry algal flakes are obtained from the plot.

Method of inoculation of BGA in rice field

Blue green algae may be applied as soil based inoculum to the rice field following the method described below.

- Powder the soil based algal flakes very well.
- Mix it with 10 kg soil or sand (10kg powdered algal flakes with 10 kg soil / sand).
- BGA is to be inoculated on 7-10 days after rice transplanting.
- Water level at 3-4" is to be maintained at the time of BGA inoculation and then for a month so as to have maximum BGA development.

Observation

A week after BGA inoculation, algal growth can be seen and algal mat will float on the water after 2-3 weeks. The algal mat colour will be green or brown or yellowish green.

Mass production and field application of *Azolla*

Azolla is a free-floating water fern that floats in water and fixes atmospheric nitrogen in association with nitrogen fixing blue green alga *Anabaena azollae*. *Azolla* fronds consist of sporophyte with a floating rhizome and small overlapping bi-lobed leaves and roots.

Rice growing areas in South East Asia and other third World countries have recently been evincing increased interest in the use of the symbiotic N2 fixing water fern *Azolla* either as an alternate nitrogen sources or as a supplement to commercial nitrogen fertilizers. *Azolla* is used as biofertilizer for wetland rice and it is known to contribute 40-60 kg N ha-1 per rice crop. The agronomic potential of *Azolla* is quite significant particularly for rice crop and it is widely used as biofertilizer for increasing rice yields.

Rice crop response studies with *Azolla* biofertilizer in the People's Republic in China and in Vietnam have provided good evidence that *Azolla* incorporation into the soil as a green manure crop is one of the most effective ways of providing nitrogen source for rice.

The utilization of *Azolla* as dual crop with wetland rice is gaining importance in Philippines, Thailand, Srilanka and India. The important factor in using *Azolla* as a biofertilizer for rice crop is its quick decomposition in soil and efficient availability of its nitrogen to rice. In tropical rice soils the applied *Azolla* mineralizes rapidly and its nitrogen is available to the rice crop in very short period. The common species of *Azolla* are *A. microphylla, A. filiculoides, A. pinnata, A. caroliniana, A. nilotica, A. rubra* and *A. mexicana*.

I. Mass multiplication of *Azolla* under field conditions

A simple *Azolla* nursery method for large scale multiplication of *Azolla* in the field has been evolved for easy adoption by the farmers.

Materials

- One cent (40 sq.m) area plot
- Cattle dung
- Super phosphate
- Furadan
- Fresh *Azolla* inoculum

Procedure

- Select a wetland field and prepare thoroughly and level uniformly.
- Mark the field into one cent plots (20 x 2m) by providing suitable bunds and irrigation channels.
- Maintain water level to a height of 10 cm.

- Mix 10 kg of cattle dung in 20 litres of water and sprinkle in the field.
- Apply 100 g super phosphate as basal dose.
- Inoculate fresh *Azolla* biomass @ 8 kg to each pot.
- Apply super phosphate @ 100 g as top dressing fertilizer on 4th and 8th day after *Azolla* inoculation.
- Apply carbofuran (furadan) granules @ 100 g/plot on 7th day after *Azolla* inoculation.
- Maintain the water level at 10 cm height throughout the growth period of two or three weeks.
- Observations
- Note the *Azolla* mat floating on the plot. Harvest the *Azolla*, drain the water and record the biomass.

II. Method of inoculation of *Azolla* to rice crop

The *Azolla* biofertilizer may be applied in two ways for the wetland paddy. In the first method, fresh *Azolla* biomass is inoculated in the paddy field before transplanting and incorporated as green manure. This method requires huge quantity of fresh *Azolla*. In the other method, *Azolla* may be inoculated after transplanting rice and grown as dual culture with rice and incorporated subsequently.

A. *Azolla* biomass incorporation as green manure for rice crop

- Collect the fresh *Azolla* biomass from the *Azolla* nursery plot.
- Prepare the wetland well and maintain water just enough for easy incorporation.
- Apply fresh *Azolla* biomass (15 t ha-1) to the main field and incorporate the *Azolla* by using implements or tractor.

***B. Azolla* inoculation as dual crop for rice**

- Select a transplanted rice field.
- Collect fresh *Azolla* inoculum from *Azolla* nursery.
- Broadcast the fresh *Azolla* in the transplanted rice field on 7th day after planting (500 kg / ha).
- Maintain water level at 5-7.5cm.

- Note the growth of *Azolla* mat four weeks after transplanting and incorporate the *Azolla* biomass by using implements or tranctor or during inter-cultivation practices.
- A second bloom of *Azolla* will develop 8 weeks after transplanting which may be incorporated again.
- By the two incorporations, 20-25 tonnes of *Azolla* can be incorporated in one hectare rice field.

4. Application of Biofertilizers

1. Seed treatment or seed inoculation
2. Seedling root dip
3. Main field application

Seed treatment

One packet of the inoculant is mixed with 200 ml of rice kanji to make a slurry. The seeds required for an acre are mixed in the slurry so as to have a uniform coating of the inoculant over the seeds and then shade dried for 30 minutes. The shade dried seeds should be sown within 24 hours. One packet of the inoculant (200 g) is sufficient to treat 10 kg of seeds.

Seedling root dip

This method is used for transplanted crops. Two packets of the inoculant is mixed in 40 litres of water. The root portion of the seedlings required for an acre is dipped in the mixture for 5 to 10 minutes and then transplanted.

Main field application

Four packets of the inoculant is mixed with 20 kgs of dried and powdered farm yard manure and then broadcasted in one acre of main field just before transplanting.

Rhizobium

For all legumes *Rhizobium* is applied as seed inoculant.

Azospirillum/Azotobacter

In the transplanted crops, *Azospirillum* is inoculated through seed, seedling root dip and soil application methods. For direct sown crops, *Azospirillum* is applied through seed treatment and soil application.

Phosphobacteria

S. No.	**Crop**	**Seed**	**Nursery**	**Seedling dip**	**Main field**	**Total requirement of packets per ha**
1.	Rice	5	10	5	10	30
2.	Sorghum	3	-	-	10	13
3.	Pearl millet	3	-	-	10	13
4.	Ragi	3	-	5	10	18
5.	Maize	3	-	-	10	13
6.	Cotton	3	-	-	10	13
7.	Sunflower	3	-	-	10	13
8.	Castor	3	-	-	10	13
9.	Sugarcane	10	-	-	36 (3 split)	46
10.	Turmeric	-	-	-	24 (2 split)	24
11.	Tobacco	1	3	-	10 g/pit	14
12.	Papaya	2	-	-	10	-
13.	Mandarin Orange	2	-	-	10 g/pit	-
14.	Tomato	1	-	-	10	14
15.	Banana	-	-	5	10 g/pit	-

Inoculated through seed, seedling root dip and soil application methods as in the case of *Azospirillum*.

Combined application of bacterial biofertilizers.

Phosphobacteria can be mixed with *Azospirillum* and *Rhizobium*. The inoculants should be mixed in equal quantities and applied as mentioned above.

Points to remember

- Bacterial inoculants should not be mixed with insecticide, fungicide, herbicide and fertilizers.
- Seed treatment with bacterial inoculant is to be done at last when seeds are treated with fungicides.

Biofertilizers recommendation (one packet - 200 g)

Rhizobium (only seed application is recommended)

S. No.	**Crop**	**Total requirement of packets per ha**
1.	Soybean	5
2.	Groundnut	5
3.	Bengalgram	5
4.	Blackgram	3
5.	Greengram	3
6.	Redgram	3
7.	Cowpea	3

Phosphobacteria

The recommended dosage of *Azospirillum* is adopted for phosphobacteria inoculation; for combined inoculation, both biofertilizers as per recommendations are to be mixed uniformly before using.

Azolla – The best feed for cattle and poultry

Azolla is a free floating water fern that floats in water and fixes nitrogen in association with the nitrogen fixing blue green algae, *Anabaena azollae*. Azolla is considered to be a potential biofertilizer in terms of nitrogen contribution to rice. Long before its cultivation as a green manure, Azolla has been used as a fodder for domesticated animals such as pigs and ducks. In recent days, Azolla is very much used as a sustainable feed substitute for livestock especially dairy cattle, poultry, piggery and fish.

Azolla contains 25 – 35 per cent protein on dry weight basis and rich in essential amino acids, minerals, vitamins and carotenoids including the antioxidant b carotene. Cholorophyll a, chlorophyll b and carotenoids are also present in Azolla, while the cyanobiont *Anabaena azollae* contains cholorophyll a, phycobiliproteins and carotenoids. The rare combination of high nutritive value and rapid biomass production make Azolla a potential and effective feed substitute for live stocks.

Inputs required

Azolla fronds, Polythene sheet, Super phosphate and Cow dung.

Methodology

The area selected for Azolla nursery should be partially shaded. The convenient size for Azolla is 10 feet length, 2 feet breadth and 1 feet depth. The nursery plot is spread with a polythene sheet at the bottom to prevent water loss. Soil is applied to a depth of 2 cm and a gram of super phosphate is applied along with 2 kg of vermicompost or cow dung in the nursery for quick growth. Azolla mother inoculum is introduced @ 5 kg/plot.

The contents in the plot are stirred daily so that the nutrients in the soil dissolve in water for easy uptake by Azolla. Azolla is harvested fifteen days after inoculation at the rate of 50-80 kg / plot. One third of Azolla should be left in the plot for further multiplication. Five kg cow dung slurry should be sprinkled in the Azolla nursery at ten days intervals. Neem oil can be sprayed over the Azolla at 0.5 5 level to avoid pest incidence.

Animal	Dosage / day
Adult cow , Buffalo, Bullock	1.5-2 kg
Layer, Broiler birds	20 – 30 grams
Goat	300 – 500 grams
Pig	1.5 – 2.0 kg
Rabbit	100 gram

Value of the technology

The egg yield is increased in layer birds due to Azolla feeding. The Azolla fed birds register an overall egg productivity of 89.0 per cent as against 83.7 per cent recorded by the birds fed with only concentrated feed. The average daily intake of concentrated feed is considerably low (106.0 g) for birds due to Azolla substitution as against 122.0 g in the control birds.

More importantly Azolla feeding shows considerable amount of savings in the consumption of concentrated feed (13.0 %) leading to reduced operational cost. By considering the average cost of the concentrated feed as Rs. 17/ Kg, a 13.0 % saving in the consumption ultimately leads to a feed cost savings of 10.0 paise /day/ bird and hence a layer unit maintaining 10,000 birds could cut down its expense towards feed to a tune of rs.1000/day.

Benefits

The Azolla feeding to layer birds increase egg weight, albumin, globulin and carotene contents. The total protein content of the eggs laid by the Azolla fed birds is high and the total carotene content of Azolla eggs(440 g 100 g-1 of edible portion)is also higher than the control. The rapid biomass production due to the high relative growth rate, increased protein and carotene contents and good digestability of the Azolla hybrid Rong ping favour its use as an effective feed supplement to poultry birds.

Effect of Azolla hybrid Rong Ping on the nutritional value of egg

Parameters	***Azolla* egg**	**Control**	**percentage increase over control**
Egg weight (g)	61.20	57.40	6.62
Albumin (g /100 g of edible portion)	3.9	3.4	14.70
Globulin (g /100 g of edible portion)	10.1	9.5	6.31
Total protein (g/ 100 g of edible portion)	14.0	12.9	8.52
Carotenes (µg / 100 g of edible portion)	440	405	8.64

Application

In Indian conditions, agriculture is very much coupled with poultry farming. Azolla is an important low cost input, which plays a vital role in improving soil quantity in sustainable rice farming. The twin potentials as biofertilizer and animal feed make the water fern Azolla as an effective input to both the vital components of integrated farming, agricultural and animalo husbandry.

Limitation

Azolla is a water fern and requires a growth temperature of 35-38° C. The multiplication of Azolla is affected under elevated temperature. Hence adopting this technology in dry zones where the temperature exceeds 40°c is difficult.

UNIT- IV

Importance of microbial fermentation - advantages - sources and uses

Fermentation is one of the oldest applied biotechnologies, having been used in food preservation for over 6,000 years. It is an inexpensive and manageable food preservation technique, and very appropriate where other processing technologies - such as canning and freezing - are either inaccessible or non-existent. Fermentation processing is also labour intensive with minimal infrastructural and energy requirements, and is well integrated into village life in rural areas of many developing countries. Fermentation enhances the nutrient content of foods through the biosynthesis of vitamins, essential amino acids and proteins, by improving protein and fibre digestibility, by enhancing micronutrient bioavailability, and by degrading antinutritional factors. It also provides a source of calories when used in the conversion of substrates, unsuitable for human consumption, to human foods. Fermentation processes enhance food safety by reducing toxic compounds such as aflatoxins and cyanogens, and producing antimicrobial factors such lactic acid, bacteriocins, carbon dioxide, hydrogen peroxide and ethanol which facilitate inhibition or elimination of food-borne pathogens. Therapeutic properties of fermented foods have also been reported. In addition to its nutritive, safety and preservative effects, fermentation enriches the diet through production of a diversity of flavours, textures and aromas. It improves the shelf-life of foods while reducing energy consumption required for their preparation.

The production of fermented foods is also important in adding value to agricultural raw materials, thus providing income and generating employment. Traditional fermentation processing is generally a spontaneous, non-aseptic operaations which result from the competitive activities of a variety of microorganisms. In a bioreactor - which may consist of clay or metal pots, a basket, or a simply hole in the ground lined with leaves - strains best adapted, and with the highest growth rates, dominate under uncontrolled conditions. Optimisation of process controls and of the microbial flora associated with fermentations therefore poses one of the biggest challenges in improving food fermentation technologies. Appropriate quality control methodologies - e.g. use of high quality raw materials, proper hygienic standards in the processing environment, proper packaging - also need to be developed.

Improvements in process control through the development of more appropriate bioreactors, particularly those suitable for solid substrate

fermentations, could improve the quality and quantity of fermented foods available in developing countries. The selection and development of more productive microbial strains, and the control and manipulation of culture conditions could also increase the efficiency of fermentation processes.

Application of microbial sources in industrial application - methods

Food fermentation covers a wide range of microbial and enzymatic processing of food and ingredients to achieve desirable characteristics such as prolonged shelf-life, improved safety, attractive flavor, nutritional enrichment, and promotion of health. Fermentation enhances the nutrient content of foods through the biosynthesis of vitamins, essential amino acids and proteins, by improving protein and fiber digestibility, by enhancing micronutrient bioavailability, and by degrading anti-nutritional factors. It also provides a source of calories when used in the conversion into human foods of substrates, which are unsuitable for human consumption. Fermentation processes also enhance food safety by reducing toxic compounds such as aflatoxins and cyanogens, and producing antimicrobial factors such lactic acid, bacteriocins, carbon dioxide, hydrogen peroxide and ethanol which facilitate inhibition or elimination of food-borne pathogens. Microorganisms are commonly used to modify, improve, stabilize food products and to generate metabolites such as acids, alcohols, amino acids, and enzymes for food use.

In general, the goal of most food fermentations is to degrade many of the fermentable sugars so there is no food remaining for harmful bacteria. Common products include citric acid (used in beverages), acetic acid, glutamic acid, and ethanol. Typically submerged cultures in which microbes are grown in a large stirred tank aim towards produce one material at a time. The approach to food fermentations is much more complex since the microbes act upon an edible material, changing the organoleptic (taste, smell, and texture) properties which often involves a complex array of compounds – some that we can easily taste and some that are below the taste threshold. The microbes involved therefore are multifunctional consuming multiple nutrient sources and generating many products. Often the microbial biomass also forms much of the product material itself. Fermented foods include cheese, bread, sauerkraut, yogurt, and many Asian foods such as meso, tempeh, and soy sauce. Fermented foods have been produced throughout history and there are common themes which have arisen in disparate parts of the world.

The commonality stem from using similar input materials (milk, for example) which is acted upon by indigenous microbes normally found on the food or acted upon by inoculated cultures added purposefully to generate a desired change in the material.

Fermentation enhances the nutrient content of foods through the biosynthesis of vitamins, essential amino acids and proteins, by improving protein and fiber digestibility, by enhancing micronutrient bioavailability, and by degrading anti-nutritional factors. It also provides a source of calories when used in the conversion into human foods of substrates, which are unsuitable for human consumption. Fermentation processes also enhance food safety by reducing toxic compounds such as aflatoxins and cyanogens, and producing antimicrobial factors such lactic acid, bacteriocins, carbon dioxide, hydrogen peroxide and ethanol which facilitate inhibition or elimination of food-borne pathogens.

The initial motivation for allowing foods to ferment lies in the preservation of food products. Essentially, benign microbes that are safe are allowed to degrade the sugars and proteins in a food so as to remove any nutrient source for pathogenic or harmful microbes. This leads to a preservation of the material while in most cases still maintaining the nutritional properties. For example, yogurt is a product of milk fermentations and can be kept in a refrigerator for 3-4 weeks whereas the milk itself will begin to spoil after 1 week.

The purpose of fermenting food is to:

1. Improve the smell, flavor, and texture
2. Destroy undesirable components
3. Enhance nutritional value
4. Reduce cooking time
5. Enhance storage longevity

The product generated will depend on the following factors:

- Starting material (substrate)
- Type of microorganism(s)
- pH and salt content
- Oxygen availability

The dynamics of growth, survival and biochemical activity of microorganisms in food are the result of so-called stress reactions in response to the physical and chemical microenvironment of a food material.

Ecological approaches to study the evolution of microbial flora can be used to comprehend the microbiological processes involved in food processing, to improve microbiological safety by monitoring in situ pathogenic bacteria, and to evaluate the effective compositions of the microbial populations.

Acid-based fermentations

The most commonly utilized food fermentations can be classified as acid fermentations since organic acids (citric, acetic, glutamic) are the primary byproducts from microbial metabolism of available sugars. Lactic acid is the most common product and is often present at the highest concentrations in foods. Not surprisingly, bacteria that produce lactic acid are termed, the lactic acid bacteria. Examples of lactic acid bacteria include: *Lactobacillus, Lactococcus, Streptococcus, Pediococcus,* and *Leuconostoc*. Normally the growth of lactic acid bacteria is stimulated by altering the environmental conditions of the food material, ideally with conditions which do not promote the growth of endogeneous spoilage microbes.

- elevated T
- anaerobic conditions
- reduced water activity (salt addition)
- lowered pH

Lactic acid bacteria are categorized based on the types of products generated in addition to lactic acid. Glucose is the usual input substrate and primary carbon source.

Homolactic fermentors (also called homofermentative)

generates one product = lactate (=lactic acid)

performed by most of the lactic acid bacteria

Heterolactic fermentation (also called heterofermentative)

generates multiple products: lactate, acetic acid, ethanol, CO_2

performed by only a few species - *bifidobacterium*

Controlled fermentations are used to generate stable products, especially when the desired microorganism might grow slowly or where pathogenic microorganisms might also grow. Since the addition of selected bacteria (usually lactic acid bacteria) starts the manufacturing process, they are commonly referred to as starter cultures. Starter cultures are added to the raw materials in large numbers and incubated under optimal conditions.

In common controlled-fermented products such as sauerkraut and yogurt, the primary function of lactic acid bacteria starters is the production of lactic acid from lactose which helps to make the products shelf-stable. Other functions of starter cultures may include adding particular types of flavor, aroma, and alcohol production; proteolytic and lipolytic activities; and inhibition of undesirable organisms.

Microbial starters are genetically stable and provide particular characteristics through fermentation in a more controlled and predictable way. Modern starter cultures are selected either as single or multiple strains, specifically for their adaptation to a substrate or raw material. Suitable cultures for fermentation must be selected at the strain level since not all strains of a species are equally suitable for use as starters, nor are all equally well adapted to a food substrate. In dairy products, the properties of starter cultures such as phage sensitivity, lactic acid, gas, and aroma compounds production are dependent upon the properties of each culture component. Because modern dairy starter cultures are blended empirically for the desired characteristics of the final product, maintenance of the optimal strain balance throughout a cheese fermentation process is important because the complex relationships among microorganisms can be easily be altered.

Small variations in microbial composition could have unexpected effects on product quality particularly since oftentimes 1% of the final processed material is comprised of the starter culture biomass. The market for dairy cultures is currently approximately US $250 million. The identity of cultures for food products (especially for alcoholic beverages) is often a closely guarded trade secret. The dynamics of growth, survival and biochemical activity of microorganisms in foods are the result of stress reactions in response to the changing of the physical and chemical conditions into the food microenvironment (e.g., the gradients of pH, oxygen, water activity, salt, and temperature) and the ability to colonize the food matrix and to grow with spatial heterogeneity (e.g., microcolonies and bio-films). Communities of any organisms are static neither in space nor time. The growth, survival, and activity of any one species or strain, whether it be an unwanted spoilage or pathogenic organism, or a desirable biocontrol of a probiotic agent, will, in most cases, be determined by the presence of other microorganisms and the in situ cell-to-cell ecological interactions which often happen in a solid phase.

It has been shown that in situ solid phase associations similar to those found in food ecosystems could induce unique biochemical and physiological reactions of a microbial population as a whole.

Understanding the patterns of occurrence of microbial populations in food is really a problem of determining the actual niche of the organism present; that is, identifying the location (in terms of resource quality, microclimate, and presence of other microorganisms) and the role of an organism in both space and time.

Reliable quantitative ecological data should take into consideration the dynamics of microorganisms in food ecosystems. This information is of key importance to understanding the behavior of pathogenic microbes in foods. Since a primary goal of fermenting foods is to prevent spoilage, we should define and explain what spoilage means. Spoilage is an alteration in the organoleptic properties of a food that decreases its desirability for consumption. This is an ambiguous definition since it depends greatly on a person's perspective. That is, one man's spoiled milk is another man's sour cream. Spoilage can involve growth of pathogenic organisms which cause disease, but their presence is not necessary for a product to be considered spoiled. Metabolic activities that lead to food spoilage include fermentation of the normally present sugars into acids and alcohols; proteolysis of proteins producing amines and sulfides, and lipolysis (lipid degradation) leading to production of fatty acids which may later be further degraded. Pickled foods (like pickles) the combination of the addition of large amounts of salt and the anaerobic formation of acids like lactic and citric acid inhibit the growth of many spoilage microbes which cannot grow in the acidified, low water activity (due to the high salt) environment.

The sub-lethally injured bacteria: Stress is any change in the genome, proteome or environment that imposes either reduced growth or survival potential. Such changes lead to attempts by a cell to restore a pattern of metabolism that either enhances survival or generates faster growth. For any stress, the bacterial cell has a defined range within which the rate of increase of colony forming units (CFUs) is positive (growth), zero (survival) or negative (death). In the first two cases the cells are sublethally injured, whereas in the case of death the cells are lethally damaged and rapidly auto-lyse. The conditions underwhich the cell moves from one physiological state into the next is conditional on the degree of stress imposed by other environmental conditions.

In microbial populations, viable cells are usually countable on both non-selective and selective media, whereas stressed cells are able to form colonies on nonselective media but are not countable on selective media.

In foods, many adverse conditions such as nutrient depletion, low temperature and other stresses can sublethally damage microorganisms leading to what is called the viable but noncultivable (VNC) state. These cells are still capable of metabolic activity but do not produce colonies on media that normally support their growth. Sub-lethally injured cells (including VNC cells) are, however, usually able to resume their healthy state once environmental conditions become more favorable. The VNC, most importantly, can be very difficult to detect although they still represent a potential hazard when consumed.

Vegetable Products

Introduction

The fermentation of vegetables, a practice that originated in the orient, has been used as a means of preserving food for more than 2000 years. In the third century B.C., large-scale production of fermented vegetables (cabbages, radishes, turnips, cucumbers, etc.) was reported during the construction of the Great Wall of China. The most important fermented vegetables found on today's market are pickles, sauerkraut, and olives. Carrots, cauliflower, celery, okra, onions, and sweet and hot peppers are also sold as fermented vegetable products.

Soybeans have been an important source of protein, fat, and flavor for oriental pie for thousands of years. Many foods are prepared from fermented soybean, including soy sauce, miso, tempeh, and natto. Vegetable products are prepared not only by bacterial fermentation but also predominantly by fungi in particular species *of Aspergillus.* In making soy sauce and miso, a koji is prepared in a preliminary fermentation that is used in a second stage to ferment a combination of cooked soybeans and cereal. The cereal is usually wheat when making soy sauce I rice when making miso. Iru, a product derived from Jocust beans fermented by *Bacillus subtilis* and *B. licheniformis,* is the most important food condiment in Nigeria.

Fermented Vegetable Products

Sauerkraut

Sauerkraut, a major fermented vegetable food in Europe and the United States, is produced from the natural lactic acid fermentation of cabbage that has been shredded and salted. The procedure for producing sauerkraut is as follows:

1. The cabbage is allowed to wilt for a day or two at room temperature.
2. The cabbage is washed carefully, and all damaged areas are removed.
3. The cabbage is finely sliced, and salt is added to a final concentration of 2.25-2.50%, being evenly distributed to avoid high salt pockets. The addition of salt purges the cabbage, and subsequently liquid containing the carbohydrate 2.9-6.4% of the total weight of the cabbage) is squeezed out. How much lactic cid will be produced during the fermentation is determined eventually by the mount of sugar present in the liquid.
4. The shredded, salted cabbage is allowed to soften for a while to prevent breakage of the shreds during packaging in the fermentation vats.
5. The shreds are packed densely but gently, and weights are applied to the surface of the cabbage until enough liquid has been extruded to cover the surface entirely.
6. Conditions are made anaerobic to prevent spoilage by yeasts and molds by placing plastic sheets over the surface of the brine and filling it with water or brine.
7. Fermentation takes place at 18.3°C or lower, since this temperature favors the growth of heterofermentative lactic acid bacteria over the other bacteria and therefore give the sauerkraut a superior taste.

At the beginning of the fermentation, some oxygen will remain in the shredded cabbage. Plant cells, aerobic bacteria, yeasts, and molds will consume this re-ling oxygen and die off, as the supply diminishes. The facultative anaerobes then increase in number. First, coliform species (e.g., *Enterobacter cloacae) Flavobacterium* species produce gas and volatile fatty acids, and start prong flavors. As the acidity increases, these organisms are normally replaced by *Leuconostoc mesenteroides,* which will become the predominant organism. This heterofermentative organism will produce lactic acid as well as acetic acid, ethanol, and mannitol esters, which impart a bitter flavor, and carbon dioxide. *Lc. mesenteroides* is also responsible for the good flavor, diacetyl, attributed to sauer-kraut. As the acidity continues to increase and reaches 0.7-1.0%, *Lc. mesenteroides* dies off and is replaced by *Lactobacillus plantarum,* a homofermentative organism that produces lactic acid as its principal end product. *Lb. plantarum* is also responsible for the degradation of the undesirable mannitol esters produced earlier by the *Leuconostoc* species. Under favorable conditions, *Lb. plantarum* produces lactic acid until an acid concentration of 1.5-2.0% is reached.

At that point the fermentation should be completed within 1-2 months, when the total acidity has reached 1.7-2.3%. The product should be stored at low temperature or pasteurized to prevent the growth of spoilage organisms and subsequent deterioration of the product.

A recent study on the role of antimicrobial proteins in the ecology of traditional fermented foods shows that nisin, which is produced by some strains of *Lactococcus lactis lactis,* might be involved in controlling the rise *of Lb. plantarum. Leuconostoc mesenteroides,* being resistant to nisin, will therefore have a competitive advantage during the first part of the fermentation. Spoilage may result from improper fermentation temperature, incorrect salt concentration, and/or improper anaerobic conditions. Too high a fermentation temperature might result in the inhibition of Lc. *mesenteroides* and the growth of *Pediococcus cerevisiae,* which produces unfavorable results.

Too low a fermentation temperature would allow *Enterobacter* and *Flavobacterium* species to remain the predominant organisms. Too long a fermentation would result in the elimination *of Lb. plantarum,* and the ascendancy *of Lb. brevis* will further increase the acidity of the sauerkraut and produce undesirable flavors and aroma. Too low a salt concentration would result in complete spoilage of the vegetable product by any anaerobes present in the cabbage. Too high a salt concentration would enable *P. cerevisiae* to grow freely, since it is highly tolerant to salt, whereas some of the undesirable organisms might break down the cell structure.

Dark brown or black sauerkraut may result from oxidation of various compounds to chromogenic compounds by undesirable bacterial enzymes. Pink sauerkraut might result from the growth of red asporogenous yeasts, which grow well in the presence of air and high concentrations of salt. Slimy sauerkraut can also result from the presence of encapsulated varieties *of Lb. plantarum;* this product can be rinsed to make it edible, although it is not acceptable for sale to the public. A good final product should be a light-colored, crispy food having 1.72-2.3% lactic acid, a pH of 3.4-3.6, and a pleasant aroma and taste produced by diacetyl.

Cucumber Pickles

There are two different processes for producing pickled cucumbers. Salt or salt stock pickles are used for sour, sweet-sour, and mixed pickles, and pickle relishes. Dill pickles are produced differently from salt stock pickles.

Salt Stock Pickles

Immature cucumbers, in good conditions, are washed and placed in barrels where they are then brined. The wash water should preferably contain chlorine dioxide, which has been found to be 10 times more efficient than chlorine in killing microorganisms in the wash water. Cucumbers should not remain in this solution for extended periods, however, since oversoaking can result in poor fermentation later. Salt stock cucumbers can be produced in one of two ways.

The *low salt method* uses a brine containing 30 salometer (or 8%) NaCl. The cucumbers are added to this brine along with 9kg of salt per 100 kg of cucumbers. Subsequently, the salt concentration is increased by 2 salometer each week till it reaches 50 salometer. The salt concentration is then increased by 1 salometer each week till it reaches 60 salometer. The salt concentration is crucial, since a concentration of less than 6% would allow the growth of spore-forming bacteria. The *high salt method* is similar except that the suspending brine contains 10.5% salt (40 salometer).

Salt is added weekly to raise the concentration by 3 salometer until 50 salometer is reached (13.2% NaCl). In both methods, the cucumbers are held down, under the surface of the brine, during the entire fermentation. Open tanks are covered by plastic sheets. The fermentation is completed within 6-9 weeks. Potassium sorbate (0.03%) may be added to acidify the brine. This, along with constant air purging, results in salt stocks with the lowest incidence of all types of bloater damage.

Dill Pickles

There are two types of dill pickle: the overnight dill and the genuine dill pickle. Both involved the addition to the fermentation vat of the herb dill. Both have a much lower salt concentration than the salt stock pickles, and both have added acetic acid. Overnight dill pickles are prepared by adding to 45 gallons of a 20 salometer (5.3% salt) brine solution, 10 pounds of cured dill weeds, 1 pound of mixed spices, a gallon of 100 grain acetic acid, and cucumbers. The fermentation takes place at 3.3°C until the lactic acid percentage has reached 0.3-0.6%. The temperature must remain low because the salt and acid concentrations are relatively low. Genuine dill pickles are prepared by adding to a 32 salometer (7.5-8.5% salt) brine solution, the same concentration of spices, but only a quart of acetic acid per 45-gallon barrel. The fermentation should take place around 16°C but it can go up to 29°C.

The fermentation should be stopped when the lactic acid concentration has reached 1.0-1.5%. The fermentation takes around 5-6 weeks at room temperature.

Now we turn to the flora involved in the fermentation of the salt pickles. Initially, organisms such as *Pseudomonas* spp., *Flavobacterium* spp., *Alcaligenes (Acromobacter)* spp., and *Bacillus* spp. will grow and multiply. These are considered to be spoilage organisms. If the salt concentration is low, other organisms, such as coliforms, *Leuconostoc mesenteroides. Streptococcus faecalis,* and *Pediococcus cerevisiae,* will also grow and produce acid in the first few days of the fermentation. Therefore, unlike the fermentation of sauerkraut, *Leuconostoc* spp. never predominate in the initial stages of the fermentation.

If the salt concentration is not too high, *Lactobacillus brevis* will begin to grow and contribute to the acidity of the fermentation fluids. In the low salt brine, a mixture of low acid tolerant species of *Leuconostoc* and the high acid tolerant species of *Lactobacillus* and *Pediococcus* will predominate during the intermediate stages of fermentation. In the final stages, *P. cerevisiae, Lb. brevis,* and *Lb. plantarum* predominate. As the salt concentration exceeds 8%, *P. cerevisiae* lose its activity and the two lactobacilli complete the fermentation.

The final product should have 0.9% lactic acid and a pH of 3.3. The flora involved in the fermentation of genuine dill pickles initially comprises the soil bacteria, which are soon inhibited by the acid produced by *Lc. mesenteroides, S. faecalis,* and *P. cerevisiae.* Subsequently, these organisms are replaced by *Lb. plantarum,* which will mainly produce lactic acid. *Lb. brevis* may contribute to the final lactic acid concentration of 1.0-1.5%.

The major problem with pickles are due to fermentative yeasts. These organisms may cause gas production inside the cucumbers resulting in *bloaters.* The major yeast species responsible for this are *Brettanomyces, Hansenula, Saccharomyces,* and *Torulopsis; Lb. brevis* also has been observed. Another type of spoilage caused by bacteria is blackening of pickles. Organisms such as *Bacillus nigrificans,* which are found on cucumbers, are able to produce hydrogen sulfide if the process water contains high concentrations of iron or copper ions. Pickles may be preserved by refrigeration or pasteurization (internal temperature of 74°C for 15 min).

Olives

Like cucumbers, olives are fruits that are classed as vegetable substances and are fermented in a similar fashion to other vegetable products. The olives are harvested when fully developed but still green.

Fermented, ripened olives also exist, but they are less popular and are processed differently. There are three major types of green fermented olives. The *Spanish* types are brined after having received a lye treatment. The green olives are submerged in a 1.25-2.0% lye solution at 15.6-21.1°C until the solution has penetrated half to three-quarters of the way toward the pit. They are then leached to remove the lye by changing the water several times. This treatment removes some of the bitterness of the olives that is due to the glucoside oleuropein. *Greek-type* olives are also subjected to a lye treatment, which is followed by an aeration step, to produce the highly colored to jet-black olives.

These olives are placed in a high salt brine of 7-10% at the beginning and gradually increased to a final 15% salt concentration. *Siciliano-type* olives are not subjected to the lye treatment but simply brined in a final low salt concentration of 7-8%. Fermentation, which is relatively fast because the sugar has not been removed by lye treatment, is stopped when the acidity reaches 0.4-0.6%.

The microbial population responsible for the fermentation of olives differs from that of sauerkraut and pickles mainly because the higher salt concentration of the brine prevents many salt-sensitive strains from growing and gives superior advantages to salt-tolerant strains. For instance, *Lc. mesenteroides,* which is not very resistant to high salt concentration, will not dominate at any stage of the fermentation.

On the other hand, *Lb. plantarum,* which is much more tolerant to salt, will be the major fermenter. Yeasts are also very salt-resistant, and if the concentration of the brine reaches 40 salometer, they become the dominant fermenting organisms and produce mainly acetic acid. If the fermentation is allowed to continue, spoilage may occur.

Olives are apparently more sensitive to spoilage than sauerkraut and pickles. The addition of a starter culture (pure culture fermentation) *of Lb. plantarum* has been suggested as a way to reduce the chances of spoilage by undesirable organisms. The maintenance of anaerobic conditions is especially important when producing fermented olives, since yeast proliferation is a major problem in this industry. Yeast, along with mold growth, causes the production of off-flavors. Gas pockets may also result from the proliferation of yeasts and other organisms. The major organisms involved in that type of spoilage are species from the genera *Saccharomyces, Hansenula, Bacillus, Aeromonas,* and *Clostridium.*

Other Bacteria-Based Vegetable Products

Other fermented vegetable products, such as cauliflower, celery, carrots, and onions mixed with pickled cucumbers, are found on the market. Depending on the region, you might also find fermented beets, okra, and mustard leaves. These vegetables are fermented in brine or by dry-salting, as with sauerkraut. Usually leafy vegetables are dry-salted, to ensure that they remain cripsy. Sliced beets, cauliflower, tomatoes, and okra tend to become slimy during fermentation because they have a relatively high sucrose content, which is transformed into dextran during the process.

A vegetable like celery will not exhibit this feature because its sugar content is much lower. The natural fermentation of these vegetables is very similar to that of sauerkraut. *Lc. mesenteroides* initiates the fermentation, which will be continued by other lactic acid bacteria, mainly *Lb. plantarum.* The successful fermentation of green peas with strains of *Pediococcus* or *Lactobacillus* added to a brine as starter cultures and the fermentation of onions with strains of pure culture *Lactobacillus* are known. The fermentation process also greatly reduces the levels of antinutritional and toxic components such as phytates, trypsin inhibitor, cyanogenic glycosides, nitrates, and nitrites found in some raw plants and vegetables (Steinkraus, 1994; Reddy and Pierson, 1994). Fermented vegetable juices are commercially available in many European countries. These juices are prepared by adding 2-3% lactic acid bacteria starter culture per total volume of vegetable juice.

Fermented Soy Products

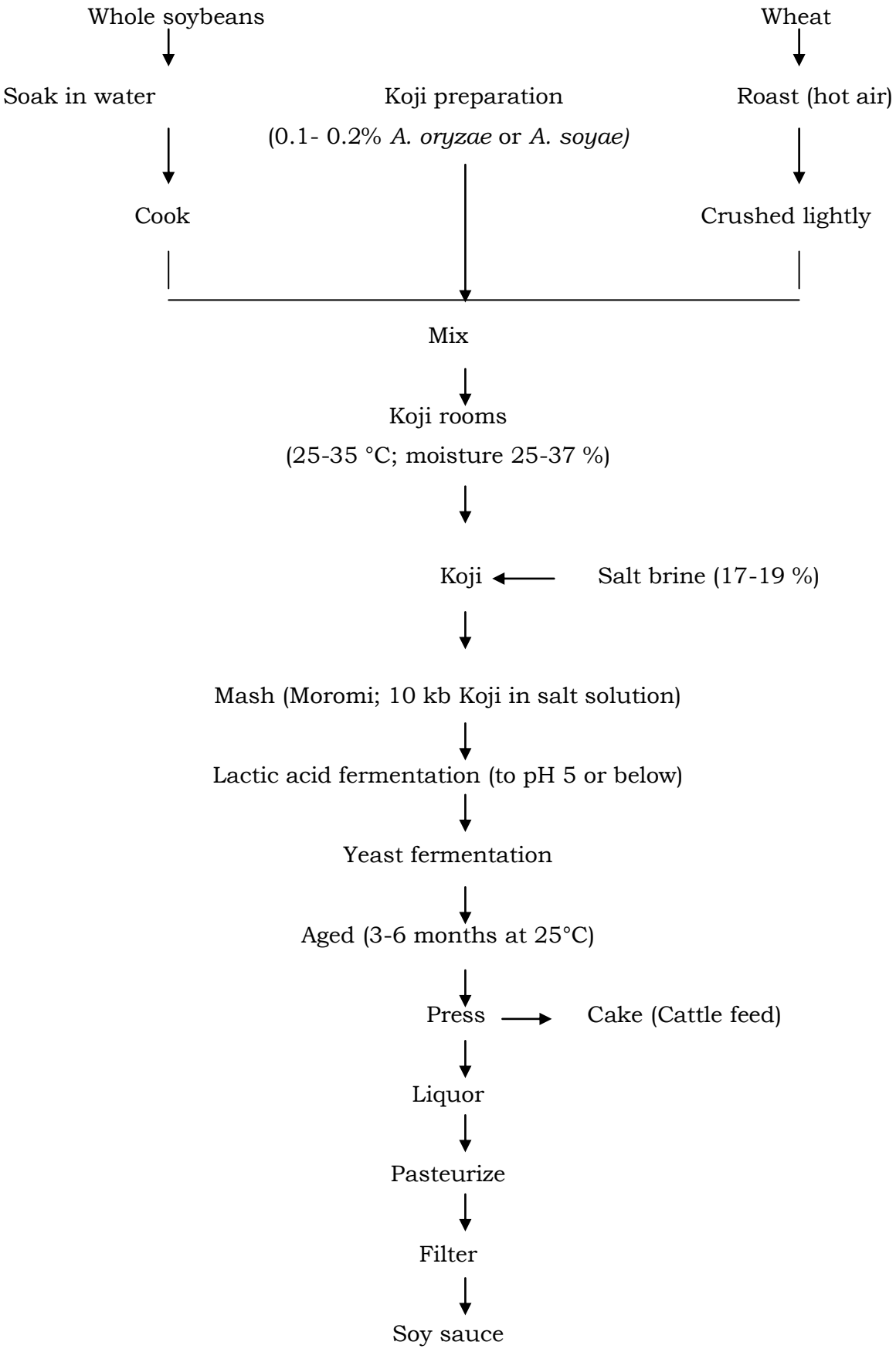
Soy Sauce

Soy sauce is a dark brown liquid, with a salty taste and a sharp flavor, which is made by fermenting soybeans, wheat, and salt. It is an all-purpose seasoning agent used in the preparation of foods as well as for a table condiment in oriental and many other countries. The fermentation of soy sauce is essentially the hydrolysis of proteins, carbohydrates, and other constituents of soybeans and wheat to peptides, amino acids, sugars, alcohols, acids, and other low molecular compounds, catalyzed by the enzymes of mold, yeast, and bacteria. In addition to the fermentation technique, two other processes are followed.

One is a chemical method, in which acid hydrolyzes the proteins and the carbohydrates, and the other is a combination of the two. Figure shows the production steps in brewing soy sauce.

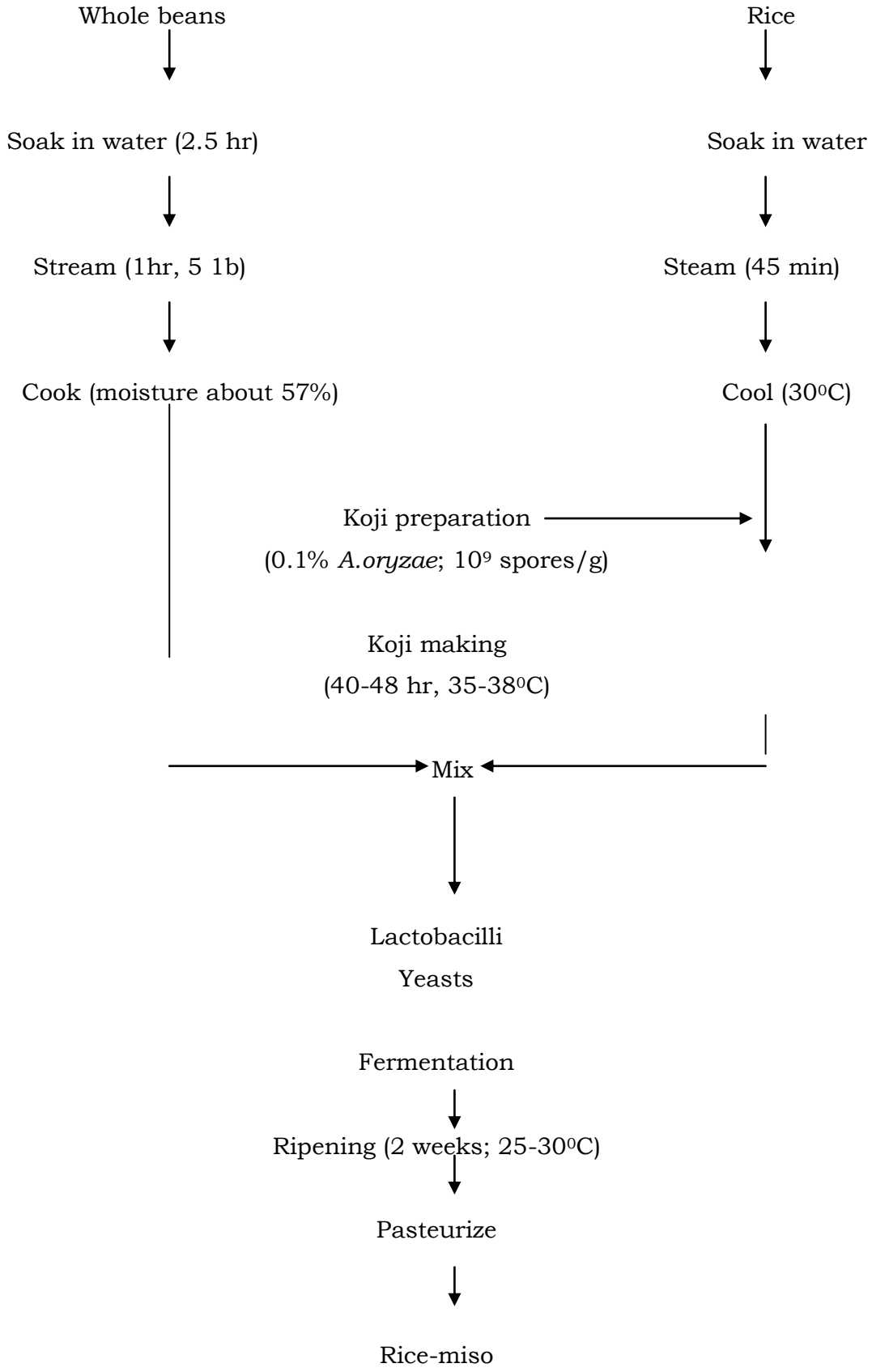
During the initial stage of mash fermentation *(moromi),* proteins and carbohydrates are hydrolyzed by *Aspergillus oryzae* or *A. soyae.* Osmophilic bacteria gradually lower the pH from about 6.7 to about 5.0. *Pediococcus soyae* is the predominant flora in the mash. In the course of low temperature fermentation, *Saccharomyces rouxii,* a dominant osmophilic yeast in the alcoholic stage of fermentation, produces about 2.5% alcohol. Yeasts and bacteria continue fermentation in the mash. A good soy sauce has a salt concentration of about 18%. Its pH is between 4.7 and 4.8; below that, the product is considered to be too acid, suggesting acid produced by undesirable bacteria. A ratio of greater than 50% of amino acid nitrogen to total soluble nitrogen is evidence of quality.

Flowchart for soy sauce manufacture



Miso

Miso, a food prepared by the fermentation of soybeans and salt with or without a cereal, is produced in a number of countries in Asia. Miso is a paste resembling peanut butter in consistency and smooth in the texture. In miso manufacture, methods differ from variety to variety, but the basic process is that charted in Figure 5.9. Briefly, it involves the cleaning and cooking of soybeans; preparation of rice koji; mixing of soybeans, salt, koji, and inoculum; fermentation under anaerobic conditions; and blending and packaging of the product for marker. Miso manufacture is essentially two successive fermentations. First koji is prepared under aerobic conditions from strains of *A. oryae* and *A.soyae*. Then follows an anaerobic fermentation involving yeasts and bacteria. During the second fermentation the enzymes convert the rice into dextrin, maltose, and glucose, which serve as fermentable sugars for the yeasts and bacteria. The soybean protein is converted to peptides and amino acids. One of the chief amino acids produced in glutamic acid, which gives miso its delicious flavour. Soybean oil is converted in part to fatty acids.



Flowchart for rice-miso manufacture

Natto

In the natural fermentation of soybeans, molds usually dominate, but one of the new products in which bacteria predominate during fermentation is *natto*, *Bacillus, natto*, also identified as *Bacillus subtilis*, is claimed to be responsible for the characteristic odor and persistent musty flavor of this organism. Natto is covered with a viscous, flavor of this organism. Natto is covered with a viscous, flavourful, and slimy glutamate polymer. There is no change in fat and fiber contents of soybeans during a 24-hour fermentation period, but the carbohydrate almost totally disappears. A great increase in water-soluble and ammoniacal nitrogen was noted during fermentation as well as during storage. The amino acid composition remained the same. Boiling markedly degreased the thiamine content of natto approximately to the same level of soybeans.

Riboflavin in natto greatly exceeds that in soybeans. Vitamin B_{12} in natto was found to be higher than in soybeans. *Soybean cooked syrup* (SCS), a by-product of natto, is rich in nutrients such as functional oligosaccharides, fibers and proteins. This product, however, has been treated as waste water and discharged by natto production companies in Japan. A study (Matsuda et al., 1992) suggested that the SCS could be used to produce a cheese like food by fermenting the syrup with lactic acid bacteria. Cultures of *Lactobacillus casei rhamnosus* were shown to be especially valuable starter bacteria for this fermentation, since this strain has an ability to decompose diazin and genistin, the SCS components that produce unpleasant tastes.

Tempeh

One of the most important fermented soybean foods, origination Indonesia, is tempeh, a cakelike product made by fermenting soybeans with *Rhizopus*. When fried in oil, it has a pleasant flavor, aroma, and texture. Unlike most of the other fermented soybean foods, which usually are used as flavor agents or relishes, tempeh serves as a main dish in Indonesia, where it constitutes a source of low cost protein.

Strains of tempeh molds produce various amounts of amylase, pectinase, lipase, and proteases. Among the strains suitable for tempeh fermentation, *Rhizopus arrhizus* NRRL 1526 appears to produce the highest amount of pectinase. All strains of *R.oligosporus* have little or no pectinase activity. Lipase is also produced by molds in tempeh fermentation.

Fatty acids are liberated by hydrolysis of soybean lipids, but there is no further utilization of these fatty acids. Either the mold does not possess the enzyme systems needed to metabolize these fatty acids or the fatty acids cannot permeate the cytoplasmic membrane of *Rhizopus*. Proteases are much more important enzymes and have an optimal pH at 3.0 (type predominating in submerged cultivation) or 5.5 (type predominating in tempeh fermentation). The enzymes are stable at H 3-6 and have high milk clotting activity.

Sufu

Sufu is a soft cheese-type product made from cubes *of soybean curd (tofu)* by the action of microorganisms. Three steps are normally involved in making sufu: preparing tofu, molding, and brining. Soybeans are first washed, soaked overnight, and then ground with water. The finely ground mixture is strained through a coarse cloth to separate the soybean milk from the insoluble residue. After the soybean milk has been heated to boiling, calcium or magnesium sulfate is added to coagulate the proteins.

The coagulated milk is then transferred into a cloth-lined wooden box and weighted atop to remove whey. A soft, but firm cakelike curd (tofu) forms. Tofu, which has a high content of water (≈ 90%), hence can be consumed directly, is eaten extensively throughout the Far East. But the water content of tofu for making sum is lower (8.3%) than that of tofu consumed directly. Otherwise, the product is likely to be spoiled by bacterial growth.

In preparation for molding, tofu is cut into small cubes (2 x 2 x 4 cm^3). The cubed are immersed in an acid saline solution of 6% sodium chloride plus 2.5% citric acid for 1 hour, arid then subjected to hot air sterilization at 100°C for 15 minutes. This treatment prevents the growth of contaminating bacteria but does not affect the growth of fungi needed in making sufu. After cooling, the cubes are inoculated over their surface with pure culture of an appropriate fungus grown on filter paper impregnated with a culture solution.

The inoculated cubes are incubated at 20°C or lower for 3-7 days, depending in the culture. The freshly molded cubes, known *as peptize,* have a luxurious growth of white mycelium and no disagreeable odor. The peptize is 74% water; 10.9% insoluble protein, 1.3% soluble protein, 4.3% liquid, and 9.5% trace components.

The last step in making sufu is brining and aging. The peptizes are placed in brining solutions of various types depending on the flavor desired. The most common brine contains 12% sodium chloride and rice wine having about 10% ethyl alcohol. The immersed cubes are allowed to age for about 40-60 days. The product is then bottled with brine, sterilized, and marketed as sufi. *Actinomucor elegans, Mucor hiemalis, M silvaticus,* and *M subtilissimus* produce sufu having a good quality, but *Actinomucor elegans* appears to be the best mold for sufu fermentation and is the one adopted commercially.

Vinegar and Other Acids

Introduction

Vinegar (acetic acid) and other organic acids are used as acidulants and flavor compounds in processed liquid foods, and as chemical feed stocks. Organic acids may be produced by chemical synthesis, by fermentation, or by extraction from natural products. Fermentative production is restricted to citric acid lactic acid. However, some acids, such as lactic acid, are also produced simultaneously by microbiological and chemical methods. Malic acid and fumaric acid are produced by fermentation but have not been used commercially owing to lack of demand.

Other acids used widely in the food industry are itaconic acid produced by *Aspergillus terreus* and gluconic acid, which is used as gluconolactone, produced by *Aspergillus niger*. Glucolactone is used as a slow acting acidulant in baking powders, and in meat processing, and so on., but gluconic acid is more readily produced by the electrochemical or enzymatic transformation of glucose. Tartaric acid produced by precipitation during wine production or by fermentation (using oxidation of malic acid by immobilized cells of *Alcaligenes levotartaricus*), is used in the food industry as an acid carrier in baking power, sweeteners, ice cream, lemonades, various fruit products and baked goods.

Except for citric acid, which is produced entirely by fermentation, there is always great competition between fermentation and chemical processes. Citric acid is by far the most important organic acid, with a world wide production exceeding 350,000 tons annually in 1982. The worldwide production of alcohol vinegar (10% acetic acid) is assumed to be about 1600 ML or 160,000 tons of pure acetic acid annually in 1980. Lactic acid has a worldwide production of about 50,000 tons and is produced by both fermentation (40%) and by chemical synthesis (60%).

Acetic Acid

Acetic acid is the main organic acid of which vinegar is composed. Traditional production of vinegar was achieved from beer or wine mash by the Orleans process, a slow surface process developed in France, in the region of Orleans. A quick process with a flowing liquid, known as the German process, was developed and became the modern tricking generator.

The Frings reactor, which is similar to the trickling generator, uses beechwood shavings bearing adhering colonies of *Acetobacter* spp. or *Gluconobacter*. The commercial strains are *Acetobacter aceti*, *A. pasteurianus*, and *A. peroxidans*. *Gluconobacter oxydans* and several subspecies are also used commercially. Ethanol based raw materials, such as wine, whey, malt, or cider, which do not require other nutrients, are trickled into the reactor from the top, and the bacteria oxidize the ethanol to acetic acid. When potato or grain spirits or technical alcohol is used, nutrients such as grain hydrolysates, ammonium phosphate, magnesium sulfate, and calcium citrate and calcium pantothenate must be added to obtain optimal growth of Acetobacter.

The raw material is fed into the reactor at the same rate at which the effluent leaves it. The residence time of the liquid depends on the rate at which ethanol is reduced to acetic acid. Mass transfer is achieved through direct contact with air that is circulating within. This technology is very similar to that based on modern immobilized cell principles, because fermentative bacteria are fixed by natural means to a carrier surface inside the reactor. The advantages of this method are low cost, higher yields, less space occupied by the tank, and low evaporation losses. Today, high-yielding strains may produce up to 13-14% acetic acid.

Further technological advance has resulted in the submerged culture process, which resembles the modern stirred-tank reactor. As in all oxidative reactions, however, oxygen availability is often the limiting factor. Acetic acid bacteria will consume on average 7.75 L of O_2 per gram of cell per hour, and less than 5% O_2 in the gas phase leads to the stoppage of the fermentation.

Although continuous processes have been described with good yields, they are very seldom used in practice. Some of the advantages of submerged cultivation over the generator are the higher efficiency of alcohol conversion, the greater holding capacity of the reactor, and low capital cost (due to fully automated operation).

Although immobilization techniques have been developed to ensure a high cell density, it is difficult to achieve consistently high viability and a high oxygen transfer rate. Since the product obtained in the submerged process in turbid because of the suspension of bacteria, the product must be filtered by plate filters and decolorized by potassium ferrocyanide. Recently recombinant DNA techniques have been used to clone the aldehyde dehydrogenase (ALDH) gene into *Acetobacter aceti* to produce transformants that have increased tolerance to acetic acid as well as greater productivity. Comparison of the acetic acid productivity of two transformants of *Acetobacter aceti* (Table 5.13) indicates that the modified *A.aceti* significantly improved the productivity and specific growth rate.

Citric Acid

Citric acid has a pleasant acid taste, is very soluble in water, and finds many applications in the food, pharmaceutical, and cosmetics industries. Citric acid occurs naturally in almost all living things and is the predominant acid in citrus fruits and in many vegetables. Originally this acid was extracted from citrus. Since 1893 scientists have known that citric acid is produced by *Penicillium glaucum*, and the first successful fermentation in surface culture was started in 1923. Fermentation processes using deep-vat fermenters began in the 1930s. Although many strains excrete traces of citric acid as primary metabolites in the tricarboxylic acid (TCA) cycle, only mutants of *Aspergillus niger* growing on carbohydrates (sucrose or molasses) or Candida lipolytica growing on paraffin substrates are used in the commercial production of citric acid. The production of undesirable side products such as oxalic acid and gluconic acid can be effectively suppressed by these mutants. The presence of metal ions in the raw materials caused drastically reduced yields and required removal by precipitation using hexacyanoferrate or by ion exchange resin (Sodeck et al., 1981). Thus, *A. niger* accumulates high concentrations of citric acid when substrate concentration is high and phosphate or metal limited. The role of metal ions in this respect is not fully under stood. Other yeasts also produce high yields of citric acid using different carbon sources such as glucose, molasses, hydrocarbons, acetate, and alcohols, but they produce a mixture of citric acid and isocitric acid. Most of the citric acid used in foods is derived from carbohydrate fermentation by *A. niger.* Small quantities (≈1%) of natural citric acid are still produced by extraction of citrus or pineapple.

COMPARISON OF ACETIC ACID PRODUCTIVITY BETWEEN TWO TRANSFORMANTS OF *ACETOBACTER ACETI*

Property	*A. aceti* (pMV24)*	*A. aceti* (pAL25)+
Acetic acid productivity (g/L/h) at 20 g/L acetate	1.8	4.0
Specific growth rate at 30 g/L acetate	0.072	0.142
Maximum acetic acid concentration, g/L	68.4	96.6

**pMV 24 is a plasmid vector developed for* Acetobacter.

+pAL 25 is the recombinant plasmid constructed by inserting a gene encoding the 75 kDa subunit of ADH complex of A. polyoxogenes *into pMV 24.*

Source: Fukara et al. (1992).

Basically two types of process are used for the commercial production of citric acid: (1) surface (koji) processes employing solid substrates (wheat bran) or liquid nutrients (sucrose, NH_4NO_3, CaH_2PO_4, $MgSO_4 \cdot 7H_2O$, $ZnSO_4$) and (2) submerged processes using stirred fermentors or air-lift fermentors. The koji process is a traditional solid state process, similar in operation to the surface process. Submerged fermentation is more difficult technically than the surface process but may be batchwise, fed-batch, or continuous in operation. At the end of the fermentation, the mycelial mass is removed by filtration and washed. Oxalate is then precipitated as calcium oxalate at a pH less than 3.0. Citrate, precipitated from the broth as tricalcium citrate tetrahydrate, is filtered off and further purified by activated carbon and ion exchange resins.

Lactic acid

Lactic acid was the first organic acid manufactured by microbial fermentation, starting in 1880 and using *lactobacilli* of three species: *delbrueckii, leishmanii,* and *bulgaricus.* This process was conducted at 45-50°C, using starch hydrolysates prepared with enzyme or acid. Today more than 50% of the lactic acid made is used in foods as an acidulant and preservative, while another 20% is used in the production of stearoyl-2-lactylates and in the pharmaceutical industry. Today only one plant, operated by the Clinton Corn Processing Company (Clinton, IA), produces lactic acid in the United States, and the main substrate is glucose obtained from starch hydrolysis. Homofermentative lactic acid bacteria are preferred because of their ability to produce lactic acid alone, with no simultaneous production of carbon dioxide and other by-products.

Thus the substrate carbon is more productively utilized. A mole of glucose is reduced to two moles of lactic acid via the EM pathway. *Lactobacillus pentosus* has been used to ferment sulfite waste liquor, while *Lb. bulgaricus* produces lactic acid from whey permeate or ammonium lactate by continuous fermentation of deproteinized whey. The lactic acid fermentation of whey has been studied in batchwise, continuous, and immobilized cell processes using different bacterial strains (Mehaia and Cheryan, 1986). *Lb. delbrueckii* produces 89 g/L/day in continuous culture, while immobilized *Lb. delbrueckii* cells in calcium alginate have been used in continuous culture, with a half-life of 100 day, yielding 90% L-(+)-lactic acid. In these processes, lactic acid is produced as the calcium salt, and thus the filtrate is treated with sulfuric acid to recover the product.

Malic Acid

Malic acid (DL-racemic mixture) is a naturally occurring acidulant, present in most fruits and vegetables. The traditional method for producing L-malic acid was by extraction from apple juice (0.4-0.7% malic acid). Malic acid finds its application as an alternative to citric acid in fruit products and lemonades. Today malic acid is produced by two additional methods: (1) chemical synthesis, via hydration of maleic or fumaric acid at high temperature and high pressure, and (2) enzymatic synthesis, whereby fumaric acid is transformed to L-malic acid. Although L-malic acid is commercially produced by fumarase from fumaric acid, it costs more than the chemically synthesized form. About 85% of the total world production of malic acid is carried out by chemical synthesis. Because of its favorable functionality in foods and competitive price, as well as new applications being developed in foods (e.g., encapsulation, mixes), this acid has the highest annual growth rate (≈ 6—8%) of the organic acids.

Malic acid fermentation by *Aspergillus* species (e.g., *A. flavus, A. parasiticus, A. oryzae, A. niger, A. wentii)* was studied in a minimal medium containing glucose, salts, and calcium carbonate (Battat et al., 1990). Among these strains, malic acid fermentation by *A. flavus* was studied in most detail. During fermentation, *A. flavus* produces unusual crystals composed of calcium malate, with minor amounts of calcium succinate and calcium fumarate. It was speculated *that A. flavus* excretes acids to the broth from the hyphae and these acids react with CaC03 present in the medium to form an insoluble calcium salt of acid residues that crystallize on the hyphae.

A. flavus is not a food-grade organism, however, and it is important that future research on malic acid production be carried out with a food-grade organism, such as *A. niger,* in which the transformation and genetic systems are better developed to improve acid accumulation. The most common commercial process in Japan is by a continuous reaction of immobilized whole cells of *Brevibacterium ammoniagenes* or *B.flavum,* where fumarase is present at a high activity. However, the L-malic acid formed is used for special purposes, thus not competing with DL-malic acid.

Fumaric Acid

Fumaric acid is also a dicarboxylic acid, and is the least expensive among the food-grade acids in terms of cost and the quantities used in foods. Fumaric acid is used similarly in the food industry as an acidulant, accounting for about 20% of the total acidulant in the U.S., and is especially suitable for using in dry beverage mixes, where its low hygroscopicity serves to extend the shelf life of other ingredients. This acid accelerates the fixation of color in cured meat and poultry products and can replace tar-taric acid in the beverage and baking industries. This acid is used not only as an acidulant but also for candy coatings, emulsifying agents, and fat and dough conditioners. It is used for the production of L-malic acid by fumarase, and L-aspartic acid by aspartase, a component of aspartame sweetener.

The major drawback of fumaric acid is its low solubility in water, and thus its application is limited mainly to dry mixes. However, a quick-dissolving product, called *cold-water-soluble* (CWS) *fumaric acid* was introduced by the Monsanto Company to overcome this problem. This product contains 0.35 (w/w) dioctyl sodium sulfosuccinate. Another product, *quick dissolving* (QD) *fumaric acid,* which contains maltodextrin carrier (4.5-6%; w/w) was introduced by Miles. In the early 1940s, fumaric acid was produced by fermentation on a commercial scale using such fungal species as *Rhizopus oryzae.* However, more attractive chemical synthesis from maleic acid has since been developed. Molar yields of fumaric acid exceed 100%, compared with malic acid (15 mol %) and succinic acid (5 mol %). The biosynthesis of fumaric acid through both the oxidative branch and part of the reductive branch of the TCA cycle may explain the high molar yield of this acid by *R. oryzae.* Three enzymes—pyruvate carboxylase, fumarase, and NAD^+-malate dehydrogenase—are localized in the cytosol and are likely to be responsible for the accumulation of malic acid.

The characterization of these enzymes and the acquisition of details of the molecular biology of *R. oryzae* are essential for the elucidation of fumarate accumulation, and for their high expression in this fungus.

Meat and Fish Products

Introduction

In meat processing, microorganisms had long been regarded as the enemy because they cause spoilage and can present a threat to public health if they are pathogenic. Fermentation is one way to prolong the shelf life of meat and fish products and has been known since ancient times. Sausages is probably the oldest form of processed food. The ancient Egyptians actually recorded the preservation of meat by salting and sun drying. The ancient Babylonians, Greeks, and Romans had also used sausage as a food source during times of war. It was not until about 1921 that it was recognized that microorganisms contribute to the production of sausages. In the 1940s and 1950s, the use of pure microbial starter cultures began, and some patents were awarded. In the 1960s commercial meat starter cultures became available to processors, and in 1968 frozen culture concentrates became commercially available for the meat industry. Use of these culture resources was not widespread until the early 1980s, however, mainly as a result of the resistance of producers, who clung to the traditional ways and feared a loss in the quality and consumer acceptance of their final product. Today the importance of the use of starter cultures is recognized in most of North America and in some European countries.

Fermented fish products constitute a major portion of the diet and increase protein intake for a large number of the world's population. The products have an amino acid composition different from that normally found in cereals and therefore complement this staple (cereals), ensuring good utilization of dietary nitrogen. In northern Europe fermented fish products are used mainly as condiments, while in areas such as Southeast Asia such products form the principal part of the diet. Evolution and development of fermented fish products in some tropical countries are due to a number of factors, including the following: fishing is encouraged in countries that have a long coastline; the provision of fresh fish to potential inland consumers may be difficult; and seasonal variations in the availability offish are typical. Salt, which has been used as a preservative since ancient times, provides a relatively easy and economical method of maintaining the fish supply.

Traditional methods have evolved, and well-defined markets are present today that are peculiar to particular regions. Some of the basic methods of preservation involve dehydration, sun drying, and pickling in brine.

Fermented Meat Products

Fermented dry sausages are defined as chopped or ground meat products that, as a result of bacterial action, reach a pH of 5.3 or less and are then dried to remove 25-50% of the moisture, resulting in a moisture-to-protein ratio no greater than 2.3:1.0. *Semidry fermented sausages,* which are the same that the moisture level is decreased by 15%, are packaged soon after the completion of the fermentation-heating process. They are generally smoked during fermentation, and the moisture-to-protein ratio must be no greater than 3.7:1.0. A review of fermented meats has been published recently (Lucke, 1994).

Classification of Fermented Sausages

In the United States, fermented sausages are classified as dry or semidry on the basis of final moisture content of the product and the descriptive terminology used in industry (Table). The European system comes from Germany (Table) and is based on the temperature treatment the product receives. Other classification systems often used in the United States are based on the ethnic origin of the sausage (Germanic, Italian, or Lebanese), the moisture-to-protein ratio, and the composition (moisture, fat, protein, salt, sugar, pH, total acidity, and yield).

CLASSIFICATION OF VARIOUS FERMENTED SAUSAGES

Class	Moisture Content (%)	Characteristics	Examples
Dry sausage	25-45%	Cured, air-dried; sometimes smoked; mold-ripened	Pepperoni Genoa salami
Semidry sausage	40-50%	Cured, air-dried; usually smoked	Lebanon bologna Summer sausage Cervelat
Fresh smoked sausage		Fresh meat, cured or uncured; smoked, not cooked; must be cooked prior to consumption	Country style Mettwurst Kielbasa
Cooked sausage		Cured or uncured; cooked and smoked	Frankfurters Bologna

Source: Adapted from Bacus (1984).

CLASSIFICATION OF FERMENTED SAUSAGES IN EUROPE (NON-HEAT TREATED)

Group	Product	Examples
Al	Raw sausage	Rohwurst
Al,* nonfermented	Fresh sausage, beef and pork	Bratwurst, Thuringer,* Numberger*
A 1, fermented	Beef and pork	Frischwurst, Mettwurst, Braunschweiger,+ Thuringer,+ Cervelat,+ Teewurst+
All, fermented	Dry sausage, salami types	Dauerwurst, Salami, Cervelat, + Plockwurst+

*Not smoked +Smoked.

Source: Adapated from Bacus (1984).

Production Process

Ingredients (%) of dry and semidry fermented sausages are:

Lean meat (pork, beef)	55—70
Fat	25-40
Curing salts	~3
Fermentable carbohydrate	0.4-2
Spices, flavorings	~0.5
Other: starter culture, ascorbic acid, nitrite	~0.5

The manufacture of dry and fermented sausages proceeds as follows. The meat is formulated by breaking (i.e., grinding, chopping, and mixing) with the fat, to give the desired fat content. This is done at cold temperatures to avoid smearing the fat. The spices, flavorings, curing salts, carbohydrate, nitrite, and starter culture are mixed in, and the mixture is stuffed into the proper sausage casings (cellulose, collagen, or natural) at a temperature of -2.2 -1.1°C. In the traditional process, there was an additional step prior to stuffing called *pan curing,* in which nitrate-reducing bacteria induced the conversion of nitrate to nitrite. Since nitrite is now added directly, this step is no longer required, and the characteristic pink color of cured meats (nitroso-hemochrome) is formed without pan curing.

After stuffing, the sausages are hung in a maturing room. It is in this room that the fermentation takes place.

The environmental conditions can vary widely during fermentation depending on the type of sausage being made. It is not the intent here to describe these conditions for the literally hundreds of different varieties of sausages. Traditionally, the temperature can range from 15.6 to 23.9°C and the relative humidity from 80 to 90%. The temperature is raised over the course of fermentation: for dry sausages, up to 37.8°C; for semidry sausages, up to 43°C. The nature of the fermentation will depend on which microorganisms are present. The dominant microorganisms in most sausages are *Lactobacillus* spp., which are generally homofermentative in that they produce only lactic acid as a product that gives the characteristic tangy flavor. Heterofermentative microorganisms can produce an assortment of end products including lactic acid, ethanol, carbon dioxide, and acetic acid, which can add to the uniqueness of a sausage; or they can be unwanted, depending on the type of product. Following fermentation, sausages are fully cooked, partially cooked, and/or placed in a drying room. Fermentation may continue during this process depending on the growth characteristics of the particular bacteria, temperature, pH, carbohydrate level, and degree of heat penetration.

Starter Cultures

To ensure products of consistent flavor, texture, and shelf stability, as well as to improve product safety, most processors have developed pure microbial cultures to control the fermentation of their sausage product. It is evident that with a starter culture, the pH drops much more rapidly, hence the whole manufacturing process is accelerated, leading to economical gains for the processor. The majority of starter cultures are natural isolates of the desirable microorganisms found in the sausage normally.

Starter cultures can come in fresh, frozen, or freeze-dried forms, and they can be single or mixed cultures of selected strains of microorganisms with definite characteristics that are beneficial in the manufacture of certain sausages. They are grown under closely controlled conditions in a liquid medium, concentrated to smaller volume, and then placed in frozen storage or other suitable medium (lyophilization) to preserve their viability and activity.

The normal inoculum level (≈10^6 organisms per gram of product) theoretically inhibits any growth of undesirable microorganisms.

A wide variety of species have been used as starter cultures and investigated for their potential for use as starter cultures

BACTERIA USED AS STARTER CULTURES IN MEAT, POULTRY AND FISH PRODUCTS

Products	Bacteria
Semidry Fermented Meat Sausages	
Lebanon bologna	Mixture of *Pediococcus cerevisiae/Lactobacillus plantarum*
Summer Sausage	*P. cerevisiae or mixture/Lb. plantarum*
Cerevelat	*P. cerevisiae or mixture/Lb. plantarum*
Thuringer	*P. cerevisiae*
Teewurst	*Lactobacillus* species
Pork roll	*P. cerevisiae*
Dry Fermented Meat Sausages	
Pepperoni	*P. cerevisiae/Lb. plantarum*
Dry sausage	*P cerevisiae*
European dry sausage	*Micrococcus species* or *Micrococcus/Lactobacillus* species
Salami	*Micrococcus/Lactobacillus* species or *Lb. plantarum*
Hard salami, Genoa	*Micrococcus* species *Micrococcus* species/***P*** *cerevisiae; Micrococcus* species/ *Lb. plantarum*
Fermented Poultry Sausages	
Semidry turkey sausage	*P. cerevisiae*
Dry turkey sausage	*P. cerevisiae or P. cerevisiae/Lb. plantarum*

SOME BACTERIA INVESTIGATED FOR THEIR POTENTIAL USE AS STARTER CULTURES

Lactobacillaceae	
Lactobacillus	Lb. plantarum, Lb. acidophilus, Lb. casei, Lb.fermenti, Lb. brevis, Lb. buchneri
Lactococcus	L. lactis, L. diacetilactis, L. acidilacti
Pediococcus	P. cerevisiae, P. acidilacti, P. pentosaceus
Micrococcaceae	
Micrococcus	M. lactis, M. auriantiacus, M. candidas, M. varians, M. epidermidis, M. conglomeratus, M. aquatilis

Source: Adapted from Bacus (1984).

The following starter culture characteristics are ideal for sausage production: salt tolerance, fast growth, nitrite tolerance, optimum temperature for growth is ~32°C, and the culture should be homofermentative, nonproteolytic, nonlipolytic, nonpatho-genic, nontoxic, and with no off-flavor production. An inactivation temperature of ~60°C is most desirable.

Starter Improvement

Culture research by genetic engineering techniques or by natural selection offers the potential for the selective elimination of such undesirable fermentation end products as excessive CO_2, while retaining the desirable flavor compounds. *Staphylococcus carnosus,* a strain routinely used for dry sausage production in Europe, is being investigated as a host strain for recombinant DNA in the genetic organization of certain staphylococci. More recently, strains *of Pediococcus cerevisiae, Pediococcus acidilacti, Lactobacillus plantarum, Lactobacillus casei,* and *Micrococcus varians* were also examined with the intent of preparing mixed cultures of compatible strains for meat fermentation. The addition of 0.18% nitrite was slightly inhibitory, while addition of 7% of a spice mix proved stimulatory to the mixed cultures. Antibiosis was observed between lactobacilli and pediococci during fermentation. Micrococci did not produce substances inhibitory toward other cultures and was insensitive to bacteriocins produced by lactobacilli or pediococci. The most rapidly fermenting strains in the mixed cultures did not give the most active culture, and mixed cultures for meat fermentation must be chosen for compatibility by means of disc assays (see chapter 9) to evaluate antibiosis between potential strains.

In the most recent research on dry fermented sausages in Spain, unexpected difficulties with starter cultures imported from northern Europe have been experienced, mainly as a result of the inhibitory action of contaminating microorganisms from different environmental conditions. Finally, two microorganisms, *Lactobacillus sake* and *Lb. curvatus,* which are insensitive to the inhibitory action of contaminated microorganisms as starter cultures, have been isolated.

New Developments

Studies of the biotechnology of fermented meat technology have lagged behind research on other fermented foods because meat is more difficult to examine than fermented fluids such as milk products and alcoholic beverages. And, practically, it is almost impossible to experiment with sterilized or pasteurized raw materials, since the effects of random microbial contaminants in raw meat cannot be eliminated. Raw meat normally contains several million contaminants per gram, and the influence of these substances on fermentation cannot be overlooked.

There are basically two paths along which development has progressed.

Commercial cultures of *Pediococcus cerevisiae* and *Micrococcus aurianticus* were simultaneously introduced to the American and European meat industries, respectively. The *Pediococcus* strains were added for their lactic acid producing capability, while the *Micrococci* were used as a control mechanism to reduce nitrates to nitrites, to effect the curing reaction. *Pediococci* do not reduce nitrate, and a mixed cure of nitrate and nitrite has replaced the traditional nitrate cure in many American processes. The Europeans depend more on chance inoculation to carry out the fermentation, and in general the fermentation times are slower, with lower temperatures being used, and thus a higher final pH. American processors use a higher temperature with a faster fermentation rate and a lower final pH. In the United States, therefore, research has focused on rapid acid-producing strains of pediococci and lactobacilli. Successful meat fermentation can now occur in as short a time as 6-8 hours, given the proper formulation, starter culture, and process controls, compared with the traditional 3-5 days. Since one effect of this rapid fermentation is the inhibition of flavor-producing microorganisms, various control mechanisms in the formulation and process are being explored to optimize the effects of both these microorganisms and the lactic acid producing bacteria.

This can be achieved to a certain extent by altering the inoculation ratio and/or varying the processing schedule to initially favor one of the lower temperature cultures (which are generally the flavor-producing ones) prior to elevating the process temperature to accelerate acid production. In Europe, research has focused on the equipment and environmental controls and a wide variety of starter cultures to effect unique flavor and color development. Also in Europe, the possibility of using starter cultures for meat products of other types (hams, dry-cured products) is being investigated.

Fermented Fish Products

In general, when fish and dry salt are packed in layers and left for a long time, cellular liquid will be extracted to form a pickle. If this is left in contact with the fish and the proteolytic enzymes of the fish are active enough, a fish sauce will develop. If the liquid is drawn off at intervals, or the period of contact is kept short, it is possible to produce a fish paste. If the period is much shorter and the amount of salt used is limited, the result will be salted fish that have undergone softening.

Three types of fermentation can occur: (1) traditional fermentation carried out by the enzymes and entrails of fresh fish in the presence of high concentrations of salt, (2) traditional fermentation carried out by the combined action of fresh fish

enzymes and entrails and microbial enzymes in the presence of salt (the microbial enzymes are added as a starter, in that they are usually present on some form of cereal-like cooked rice or maize), and (3) nontraditional processes in which products are obtained by accelerating the rate of fermentation with enzymes or by chemical hydrolysis.

Fish Sauces

Fermented fish sauces have been consumed since ancient times, and the earliest reported is *garum,* which was highly prized in the Roman era. This product was made from the blood and viscera of mackerel, and fermentation was due to the proteolytic enzymes from the viscera. Garum is thought to be different from the sauces produced today in that it was decanted to give a clear liquid, and the residue was made into a thicker sauce called *alec.* In Southeast Asia, the use of fermentation as a method of preserving fish has been of importance since earliest times. Table 5.11 lists some of the different fish sauces found in the world, along with the basic process and fish species used.

The uneviscerated fish is normally mixed with salt in the ratio of 2:1 or 5:1 (fish : salt), and is then fermented in tanks buried in the ground for at least 6 months.

FISH SAUCES

Name	**Fish Species**	**Ratio of fish to salt; fermentation time**	**Country**
Shottsur	*Astroscopus* spp. (sandfish)	5:1 salt + malted rice and koji (3:l) 6 months	Japan
Uwo-shoyu	*Clupea* spp. (sardine)		Korea
Ika-shoyu	*Omnastrephis* spp. (squid)		Hong Kong
Saewu-jot	Shrimp	4:1 salt, 6 months	Thailand
	Sardinella spp., *Jelio* spp., *Carangidae* spp., *Engraulis* spp., *Teuthis* spp.	4:1 salt, 3-12 months	
Nam-pla	*Stolephorus* spp., *Ristrelliger* spp., *Cirrhinus* spp.	5:1 -3:1 salt, 5-12 months	
Ketjap-Ikan	*Stolephorus* spp., *Clupea* spp., *Leiognathus* spp.	3:1-4:1 salt, 3-12 months	Indonesia
Budu	*Stolephorus* spp.	5:1 -3:1 salt + palm sugar and tamarind, 3-12 months	Malaysia
Patis/Bagoong	*Stolephorus* spp., Clupea spp., Bagoong *Decapterus* spp., *Leiognathus* spp.	3:1-4:1 salt, 3-12 months	Philippines

Nuoc-man/ Mam	*Stolephorus* spp., *Ristrelliger* spp. *Engraulis* spp., *Decapterus* spp., *Dorosoma* spp., *Clupea* spp.	3:1-3:2 salt, 3-12 months	Cambodia/ Vietnam
Colombo-cure	*Ristrelliger* spp., *Cybium* spp., *Clupea* spp.	Gutted fish with gills removed and tamarind added, 6:1 salt, 12 months	India and Pakistan
Garos	*Scomber colias*	Liver only, 9:1 salt, 8 days	Greece
Pissala	*Aphya pellucida, Gobius* spp., *Engraulis* spp., *Atherina* spp., *Meletta* spp.	4:1 salt, 2-8 weeks	France
Anchovy	*Engradulidae encrasicholus*	Beheaded and gutted fish, 2:1 salt, 6-7 months	Many

The supernatant liquid is drained off, filtered, and ripened for about 3 months under the sun to obtain better aroma and color.

The finished product is a liquid protein which contains a good source of amino nitrogen, Ca, P, Fe, other organic nutrients, and vitamin B complex.With traditionally produced sauces, the most important factors in consumer acceptability are flavor and aroma. Aseptically produced sauces do not produce the aroma typically associated with the respective sauces, and thus it was proposed that microorganisms were involved. The proteolytic enzymes responsible for the protein degradation that occurs may have endogenous or microbial origins. Some earlier studies have provided conflicting results and further study is needed in this area. Among the large numbers of bacteria and yeasts present in fish fermentation, microflora such as *Bacillus, Micrococcus, Lactobacillus,* and *Pediococcus genera* are most common. The high concentration of salt limits microbial growth, particularly of pathogens, and therefore reduces spoilage. The surviving microflora must thus be at least halotolerant or halophilic bacteria. These halophilic bacteria appear to be involved in the development of aroma, since the aseptically produced fish sauces did not give the typical aroma associated with them (Beddows, 1985). The production cycle for fish sauce is long, and thus many attempts have been made to speed up the production time by adding exogenous enzymes such as brome-lain, ficin, papain, trypsin, and microbial proteases. The organoleptic properties of fish sauces with added enzymes are not satisfactory, however.

Others have accelerated production offish sauces by adding some halophilic *Bacillus* C_1 or lactic acid bacteria (Steinkraus, 1995). Recently, research has focused on the chemical components of fish sauces produced in various parts of Japan (Steinkraus, 1995). The chemical components offish Sauces compare to those of fermented plant materials. There is a similarity in that both contain salty and amino acid substances as well as the umami amino acids. Other works (Lucke and Earnshaw, 1991) explored the potential use of starter cultures for the production of chilled fish products. *Leuconostoc* spp. and *Lactobacillus plantarum* showed a wide inhibitory ranges among 61 isolates of lactic acid bacteria, which were phenotypically identified and characterized based on gas production, carbohydrate fermentation, production of off-odors, peroxide production, antagonistic activity, and growth at 2, 5, and 10°C.

Fish Pastes

Much more widely produced and eaten than fish sauces, fish pastes are usually consumed in small quantities, as condiments with rice dishes.

The process of fermentation is much shorter than for sauces, and in addition, larger fish species can be used as well as fish that is too low in enzyme activity to be suitable for fish sauce production. Table 5.12 shows some of the fish pastes that have their origins in Asia.

Very few data are available on the role, if any, of microorganisms in the production of fish pastes. Whether they assist in the breakdown of tissue or in the development of flavor and aroma is a subject of speculation.

FISH PASTES AND THEIR INGREDIENTS

Name	Ingredients	Country
Kapi Pla-mam Pla-chao Kung-chao	Marine shrimp, salt Freshwater fish, salt, roasted rice, pineapple Freshwater fish, salt, glutinous rice, KNO_3 Marine or freshwater shrimp, salt, color, roasted rice, sesame	Thailand
Blachan	Shrimp, salt	Malaysia
Bagoong	Fish or shrimp, salt, color	Philippines
Trassi	Fish, salt; sun dried	Indonesia
Nga-Ngapi	Fish, salt	Burma
Shiokara Sidal	Squid or skipjack; salt; mustard rice Small fish, salt; dried, crude fish oil	Japan Pakistan and India

Salted Fish

A number of salted fish products are prepared in a way that facilitates microbial and biochemical action. The degree of proteolysis is much less than for fish paste production. In Japan, a product is made from sea bream and sandfish, which are gutted and cured with 20-30% salt. The fish are mixed with a fermenting cereal such as rice, and lactic acid bacteria thrive to lower the pH over a month or two. The fish is then desalted and the liquid is drained off. Boiled rice and koji are mixed in and a secondary fermentation occurs, giving the characteristic flavor of the product, as the yeasts in the koji become very active. In this case, lactic acid aids in preservation, and the bulk of the flavor comes from the koji and secondary fermentation.

The production of anchovies in certain countries usually involves some fermentation. The fish are gutted and beheaded and placed in a vat with salt. The contents are weighed down to extract the pickle as it forms, and the fish are matured for 6-7 months at ~16°C.

FERMENTED DAIRY PRODUCTS

Fermented milk products or fermented dairy products, also known as cultured dairy foods, cultured dairy products, or cultured milk products, are dairy foods that have been fermented with lactic acid bacteria such as Lactobacillus, Lactococcus, and Leuconostoc. The fermentation process increases the shelf life of the product while enhancing its taste and improving the digestibility of its milk. There is evidence that fermented milk products have been produced since around 10,000 BC. A range of different Lactobacilli strains has been grown in laboratories allowing for many cultured milk products with different flavors and characteristics.

Products

Many different types of cultured milk products can be found around the world including milk, cheese, yogurt, other cultured dairy foods, ice cream and more.

COMPARISON CHART

Product	Alternative names	Typical milkfat content	Typical shelf life at 4 °C	Fermentation agent	Description
Acidophilus milk	acidophilus cultured milk	0.5-2%	2 weeks	Lactobacillus acidophilus	Thermophilic fermented milk, often lowfat (2%, 1.5%) or nonfat (0.5%), cultured with Lactobacillus acidophilus
Cheese		1-75%	varies	a variety of bacteria or mold	Any number of solid fermented milk products.
Crème fraîche	creme fraiche	30-40%	10 days	naturally occurring lactic acid bacteria in cream	Mesophilic fermented cream, originally from France; higher-fat variant of sour cream
Cultured buttermilk		1–2%	10 days	Lactococcus lactis* (Lactococcus lactis subsp. lactis*, Lactococcus lactis subsp. cremoris, Lactococcus lactis biovar. diacetylactis and Leuconostoc mesenteroides subsp. cremoris)	Mesophilic fermented pasteurized milk
Cultured sour cream	sour cream	14–40%	4 weeks	Lactococcus lactis subsp. lactis	Mesophilic fermented pasteurized cream with an acidity of at least 0.5%. Rennet extract may be added to make a thicker product. Lower fat variant of crème fraîche
Filmjölk	fil	0.1-4.5%	10–14 days	Lactococcus lactis and Leuconostoc	Mesophilic fermented milk, originally from Scandinavia
Kefir	kephir, kewra, talai, mudu kekiya, milkkefir, búlgaros	0-4%	10–14 days	Kefir grains, a mixture of bacteria and yeasts	A fermented beverage, originally from the Caucasus region, made with kefir grains; can be made with any sugary liquid, such as milk from mammals, soy

					milk, or fruit juices
Kumis	koumiss, kumiss, kymys, kymyz, airag, chigee	4%?	10–14 days	Lactobacilli and yeasts	A carbonated fermented milk beverage traditionally made from horse milk
Viili	filbunke	0.1-3.5%	14 days	Lactococcus lactis subsp. cremoris, Lactococcus lactis* biovar. diacetylactis, Leuconostoc mesenteroides subsp. cremoris and Geotrichum candidum	Mesophilic fermented milk that may or may not contain fungus on the surface; originally from Sweden; a Finnish specialty
Yogurt	yoghurt, yogourt, yoghourt	0.5–4%	35–40 days	Lactobacillus bulgaricus and Streptococcus thermophilus	Thermophilic fermented milk, cultured with Lactobacillus bulgaricus and Streptococcus thermophilus

Yogurt

Yogurt (also spelled yogourt or yoghurt) is a semi-solid fermented milk product which originated centuries ago in Bulgaria. It's popularity has grown and is now consumed in most parts of the world.

Although the consistency, flavour and aroma may vary from one region to another, the basic ingredients and manufacturing are essentially consistent:

Ingredients

Although milk of various animals has been used for yogurt production in various parts of the world, most of the industrialized yogurt production uses cow's milk. Whole milk, partially skimmed milk, skim milk or cream may be used. In order to ensure the development of the yogurt culture the following criteria for the raw milk must be met:

- low bacteria count
- free from antibiotics, sanitizing chemicals, mastitis milk, colostrum, and rancid milk
- no contamination by bacteriophages

Other yogurt ingredients may include some or all of the following:

Other Dairy Products: concentrated skim milk, nonfat dry milk, whey, lactose. These products are often used to increase the nonfat solids content
Sweeteners: glucose or sucrose, high-intensity sweeteners (e.g. aspartame)
Stabilizers: gelatin, carboxymethyl cellulose, locust bean Guar, alginates, carrageenans, whey protein concentrate
Flavours
Fruit Preparations: including natural and artificial flavouring, colour

Starter Culture

The starter culture for most yogurt production in North America is a symbiotic blend of *Streptococcus salivarius* subsp. *thermophilus* (ST) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB). Although they can grow independantly, the rate of acid production is much higher when used together than either of the two organisms grown individually. ST grows faster and produces both acid and carbon dioxide. The formate and carbon dioxide produced stimulates LB growth. On the other hand, the proteolytic activity of LB produces stimulatory peptides and amino acids for use by ST. These microorganisms are ultimately responsible for the formation of typical yogurt flavour and texture. The yogurt mixture coagulates during fermentation due to the drop in pH. The streptococci are responsible for the initial pH drop of the yogurt mix to approximately 5.0. The lactobacilli are responsible for a further decrease to pH 4.0.

The following fermentation products contibute to flavour**:**

- lactic acid
- acetaldehyde
- acetic acid
- diacetyl

Manufacturing Method

The milk is clarified and separated into cream and skim milk, then standardized to acheive the desired fat content. The various ingredients are then blended together in a mix tank equipped with a powder funnel and an agitation system. The mixture is then pasteurized using a continuous plate heat exchanger for 30 min at 85° C or 10 min at 95° C. These heat treatments, which are much more severe than fluid milk pasteurization, are necessary to acheive the following:

- produce a relatively sterile and condusive environment for the starter culture
- denature and coagulate whey proteins to enhance the viscosity and texture

The mix is then homogenized using high pressures of 2000-2500 psi. Besides thoroughly mixing the stabilizers and other ingredients, homogenization also prevents creaming and wheying off during incubation and storage.

Stability, consistency and body are enhanced by homogenization. Once the homogenized mix has cooled to an optimum growth temperature, the yogurt starter culture is added.

A ratio of 1:1, ST to LB, inoculation is added to the jacketed fermentation tank. A temperature of 43° C is maintained for 4-6 h under quiescent (no agitation) conditions. This temperature is a compromise between the optimums for the two micoorganisms (ST 39° C; LB 45° C). The titratable acidity is carefully monitored until the TA is 0.85 to 0.90%. At this time the jacket is replaced with cool water and agitation begins, both of which stop the fermentation. The coagulated product is cooled to 5-22° C, depending on the product. Fruit and flavour may be incorporated at this time, then packaged. The product is now cooled and stored at refrigeration temperatures (5° C) to slow down the physical, chemical and microbiological degradation.

Yogurt Products

There are two types of plain yogurt:

- Stirred style yogurt
- Set style yogurt

The above description is essentially the manufacturing procedures for stirred style. In set style, the yogurt is packaged immediately after inoculation with the starter and is incubated in the packages. Other yogurt products include:

Fruit-on-the-bottom style:

fruit mixture is layered at the bottom followed by inoculated yogurt, incubation occurs in the sealed cups

Soft-serve and Hard Pack frozen yogurt

Continental, French, and Swiss:

stirred style yogurt with fruit preparation

Yogurt Beverages

Drinking yogurt is essentially stirred yogurt which has a total solids content not exceeding 11% and which has undergone homogenization to further reduce the viscosity, Flavouring and colouring are invariably added. Heat treatment may be applied to extend the storage life.

HTST pasteurization with aseptic processing will give a shelf life of several weeks at 2-4°C, which UHT processes with aseptic packaging will give a shelf life of several weeks at room temperature.

Cultured Buttermilk

This product was originally the fermented byproduct of butter manufacture, but today it is more common to produce cultured buttermilks from skim or whole milk. The culture most frequently used in S. lactis, , perhaps also spp. cremoris. Milk is usually heated to 95°C and cooled to 20-25°C before the addition of the starter culture. Starter is added at 1-2% and the fermentation is allowed to proceed for 16-20 hours, to an acidity of 0.9% lactic acid. This product is frequently used as an ingredient in the baking industry, in addition to being packaged for sale in the retail trade.

Acidophilus milk

Acidophilus milk is a traditional milk fermented with Lactobacillus acidophilus (LA), which has been thought to have therapeutic benefits in the gastrointestinal tract. Skim or whole milk may be used. The milk is heated to high temperature, e.g., 95°C for 1 hour, to reduce the microbial load and favour the slow growing LA culture. Milk is inoculated at a level of 2-5% and incubated at 37°C until coagulated. Some acidophilus milk has an acidity as high as 1% lactic acid, but for therapeutic purposes 0.6-0.7% is more common.

Another variation has been the introduction of a sweet acidophilus milk, one in which the LA culture has been added but there has been no incubation. It is thought that the culture will reach the GI tract where its therapeutic effects will be realized, but the milk has no fermented qualities, thus delivering the benefits without the high acidity and flavour, considered undesirable by some people.

Sour Cream

Cultured cream usually has a fat content between 12-30%, depending on the required properties. The starter is similar to that used for cultured buttermilk. The cream after standardization is usually heated to 75-80°C and is homogenized at >13 MPa to improve the texture. Inoculation and fermentation conditions are also similar to those for cultured buttermilk, but the fermentation is stopped at an acidity of 0.6%.

Others

There are a great many other fermented dairy products, including kefir, koumiss, beverages based on bulgaricus or bifidus strains, labneh, and a host of others.

Many of these have developed in regional areas and, depending on the starter organisms used, have various flavours, textures, and components from the fermentation process, such as gas or ethanol.

Probiotics

Probiotics are usually defined as microbial food supplements with beneficial effects on the consumers. Most probiotics fall into the group of organisms' known as lactic acid-producing bacteria and are normally consumed in the form of yogurt, fermented milks or other fermented foods. Some of the beneficial effect of lactic acid bacteria consumption include: (i) improving intestinal tract health; (ii) enhancing the immune system, synthesizing and enhancing the bioavailability of nutrients; (iii) reducing symptoms of lactose intolerance, decreasing the prevalence of allergy in susceptible individuals; and (iv) reducing risk of certain cancers. The mechanisms by which probiotics exert their effects are largely unknown, but may involve modifying gut pH, antagonizing pathogens through production of antimicrobial compounds, competing for pathogen binding and receptor sites as well as for available nutrients and growth factors, stimulating immunomodulatory cells, and producing lactase. Selection criteria, efficacy, food and supplement sources and safety issues around probiotics are reviewed.

Recent scientific investigation has supported the important role of probiotics as a part of a healthy diet for human as well as for animals and may be an avenue to provide a safe, cost effective, and 'natural' approach that adds a barrier against microbial infection.

Cabbage fermentation, sauerkraut, changes and preservation

Technique

Cabbage as a commodity may be preserved in its natural state for a short period of time (3 to 4 months) or it can be subjected to bacterial fermentation, controlled with salt. During fermentation acid is developed and acts as a preservative in addition to developing a desired flavor. Sauerkraut is the German word describing this fermented, salted, shredded cabbage.

By definition, sauerkraut is "acidic cabbage." It is the result of a natural fermentation by bacteria indigenous to cabbage in the presence of 2 to 3% salt. The fermentation yields lactic acid as the major product. This lactic acid, along with other minor products of fermentation, gives sauerkraut its characteristic flavor and texture.

In the production of sauerkraut, mature cabbage heads are washed and shredded. The salt is mixed with the shredded cabbage to a final concentration of about 2.5%. The salted cabbage is then tightly packed into a tierce or crock. The cabbage is protected from air (oxygen) in a manner that will permit gases produced during the fermentation to escape. A temperature of about 70°F is preferred for the fermentation. About five weeks is required for a complete fermentation.

The salting of the cabbage serves two major purposes. First, it causes an osmotic imbalance which results in the release of water and nutrients from the cabbage leaves.

The fluid expelled is an excellent growth medium for the microorganisms involved in the fermentation. It is rich in sugar and growth factors. Second, the salt concentration used inhibits the growth of many spoilage organisms and pathogens. It does not, obviously, inhibit the desired floral succession. As cabbage is approximately 90% water and the salt is dissolved entirely in the water, the actual salt concentration (brine strength) experienced by the microorganisms in their aquatic milieu is around 2.8%. Thorough and even distribution of the salt is critical. Pockets of low or high salt concentration would result in spoilage and/or lack of the desired fermentation.

Throughout the fermentation, it is critical that oxygen be excluded. The presence of oxygen would permit the growth of some spoilage organisms, particularly the acid-loving molds and yeasts.

As no starter cultures are added to the system, this is referred to as a wild fermentation. The normal flora of the cabbage leaves is relied upon to include the organisms responsible for a desirable fermentation, one that will enhance preservation and organoleptic acceptability. The floral succession is governed mainly by the pH of the growth medium.

Initially, a coliform starts the fermentation. Coliforms which have contributed to our lab-made sauerkraut in recent years have included *Klebsiella pneumoniae*, *K. oxytoca* and *Enterobacter cloacae*. As acid is produced, an environment more favorable for *Leuconostoc* is quickly formed. The coliform population declines as the population of a strain of *Leuconostoc* builds. As *Leuconostoc* is a heterofermentative lactic acid bacterium, much gas (carbon dioxide) accompanies the acid production during this stage. The pH continues to drop, and a strain of *Lactobacillus* succeeds the *Leuconostoc*. (On occasion a strain of *Pediococcus* arises instead of *Lactobacillus*.)

The complete fermentation, then, involves a succession of three major groups or genera of bacteria, a succession governed by the decreasing pH.

Bread - dough fermentation - methods - baking and quality assessment

Yeast is the driving force behind fermentation, the magical process that allows a dense mass of dough to become a well-risen loaf of bread. And yet yeast is nothing more than a single-celled fungus. How does it do it? Yeast works by consuming sugar and excreting carbon dioxide and alcohol as byproducts. In bread making, yeast has three major roles. Most of us are familiar with yeast's leavening ability. But you may not be aware that fermentation helps to strengthen and develop gluten in dough and also contributes to incredible flavors in bread.

Yeast makes dough rise: Yeast cells thrive on simple sugars. As the sugars are metabolized, carbon dioxide and alcohol are released into the bread dough, making it rise

The essentials of any bread dough are flour, water, and of course yeast. As soon as these ingredients are stirred together, enzymes in the yeast and the flour cause large starch molecules to break down into simple sugars. The yeast metabolizes these simple sugars and exudes a liquid that releases carbon dioxide and ethyl alcohol into existing air bubbles in the dough.

If the dough has a strong and elastic gluten network, the carbon dioxide is held within the bubble and will begin to inflate it, just like someone blowing up bubblegum. As more and more tiny air cells fill with carbon dioxide, the dough rises and we're on the way to leavened bread. Yeast strengthens bread dough When you stir together flour and water, two proteins in the flour -- glutenin and gliadin -- grab water and each other to form a bubblegum-like, elastic mass of molecules that we call gluten. In bread making, we want to develop as much gluten as we can because it strengthens the dough and holds in gases that will make the bread rise. Once flour and water are mixed together, any further working of the dough encourages more gluten to form. Manipulating the dough in any way allows more proteins and water to find each other and link together. If you've ever made homemade pasta, you know that each time you roll the dough through the machine, the dough becomes more elastic; in other words, more gluten is developed. And with puff pastry dough, every time you fold, turn, and roll the dough, it becomes more elastic. Yeast, like kneading, helps develop the gluten network.

With every burst of carbon dioxide that the yeast releases into an air bubble, protein and water molecules move about and have another chance to connect and form more gluten. In this way, a dough's rising is an almost molecule-by-molecule kneading. Next time you punch down bread dough after its first rise, notice how smooth and strong the gluten has become, in part from the rise.
At this stage, most bakers stretch and tuck the dough into a round to give it a smooth, tight top that will trap the gases produced by fermentation. Then they let this very springy dough stand for 10 to 15 minutes. This lets the gluten bonds relax a little and makes the final shaping of the dough easier. This rounding and resting step isn't included in many home baking recipes, but it's a good thing to do.
Fermentation generates flavor in bread
As Harold McGee, the author of *On Food & Cooking,* has pointed out, big molecules in proteins, starches, and fats don't have much flavor, but when they break down into their building blocks -- proteins into amino acids, starches into sugars, or fats into free fatty acids -- they all have marvelous flavors. Fermentation, whether it's acting on fruit juices to make wine or on flour to make bread, does exactly that -- it breaks down large molecules into smaller, flavorful ones.

This loaf of artisan bread owes its complex flavor to a lengthy fermentation, which breaks down big molecules into smaller flavorful ones.

At the beginning of fermentation, enzymes in the yeast start breaking down starch into more flavorful sugars. The yeast uses these sugars, as well as sugars already present in the dough, and produces not only carbon dioxide and alcohol but also a host of flavorful byproducts such as organic acids and amino acids. A multitude of enzymes encourages all kinds of reactions that break big chains of molecules into smaller ones -- amylose and maltose into glucose, proteins into amino acids.

As fermentation proceeds, the dough becomes more acidic. This is due in part to rising levels of carbon dioxide, but there are also more flavorful organic acids like acetic acid (vinegar) and lactic acid being formed from the alcohol in the dough. (This is similar to what happens to a bottle of wine that has been left uncorked for a while: the alcohol combines with oxygen to make vinegar.)

The acidity of the dough causes more molecules to break down. The dough becomes a veritable ferment of reactions. Eventually, the amount of alcohol formed starts to inhibit the yeast's activity.

Yeast has help in producing flavorful compounds. Bacteria are important flavor builders as well. There are bacteria in the dough from the beginning, but as long as the yeast is very active, it consumes sugars as quickly as they're produced, leaving no food for the bacteria, which also like sugar. But when bakers chill a dough and slow down its rise, the cold dramatically reduces yeast activity. The bacteria, on the other hand, function well even in cold temperatures, so they now have an opportunity to thrive, producing many more marvelously flavorful acids.

Fermented beverages

Wine is a fermented fruit juice mostly made from grapes. In wine processing, sugar is converted into alcohol in the presence of yeast. If the amount of alcohol is relatively low, the result is wine. If the alcohol content is high, the result is distilled liquor, something like gin or vodka.

However, fermentation cannot increase the alcohol level by more than 16% as yeast dies at this level and freezes the process of fermentation. Therefore higher level of alcohol is achieved through a different process of distillation. There are three types of wine. These are red wine, pink wine (also known as rose or blush) and white wine. Red wine is an alcoholic fruit drink of 10 to 14% alcoholic strength. The colour ranges from a light red to deep dark red. The varieties of grapes suitable for red wine are Cabernet Sauvignon, Merlot, Shiraz, Sauvignon Blank, Zinfandel, Pinot Noir etc. Red wines are made when the crushed grape skin pulp and seeds of purple or red varieties are allowed to remain with the juice during fermentation period. Since the inside of a grape is more or less white, red grapes can also make white wine. White wines can be produced from pigmented grapes by removal of skins, pulps and seeds before juice fermentation. The distinct flavour of grape wine originates from the grapes as raw material and subsequent processing operations. The grapes contribute trace elements of many volatile substances (mainly tarpenes) which give the final product the distinctive fruity character. In addition, they contribute non - volatile compounds (tartaric and malic acids) which impact on flavour and tannins which give bitterness and astringency.

The latter is more prominent in red wines as the tannin components are located in the grape skins.

Grape Wine Processing:

The manufacture of grape wine involves following basic steps:

- Crushing of Grapes
- Alcoholic Fermentation
- Malolactic Fermentation, if Required
- Bulk Storage and Maturation of Wine
- Racking/Clarification and Packaging

Ripe and undamaged grapes are first crushed to yield the clear juice. Maceration (the time spent while skins and seeds are left with the juice) will go on for a few hours to a few weeks. The skins, pulp and seeds are retained in the juice (musts) for making red wines as these are important source of tannins which affect the taste of wine and maturity through aging. The skins also determine the colour of the wine.

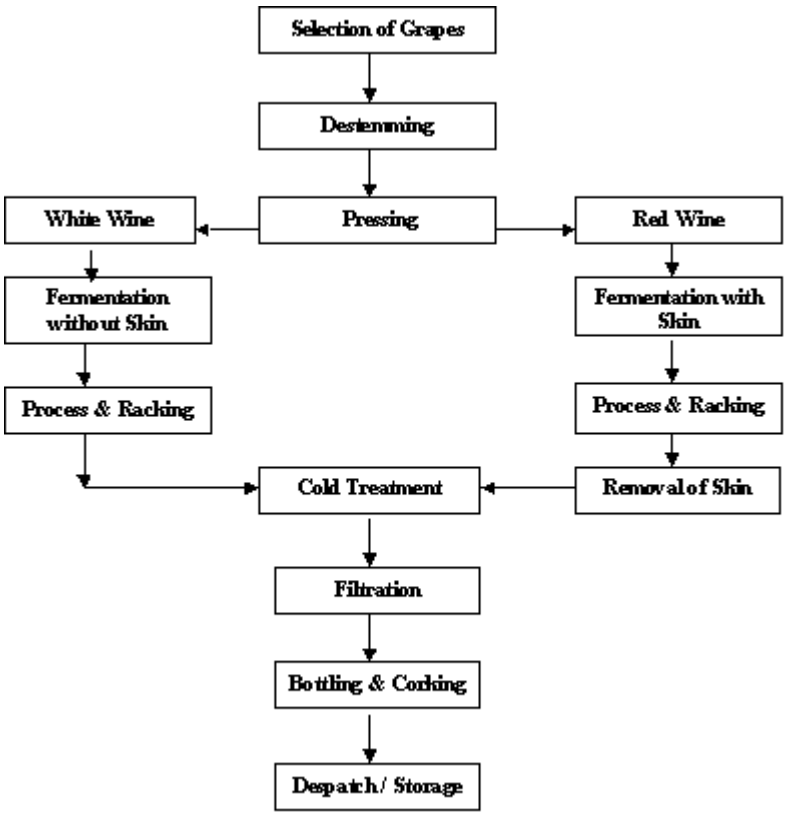
However, these parts are separated from the juice for manufacturing white wines. The musts are then pressed to extract the clear grape juice. Pneumatic Press (a cylindrical container having bladders/bags that have inflated and deflated movements) are being used to squeeze the musts until all the juice has free run leaving behind the rest of grapes.

The musts/juice is then passed through a heat exchanger to reduce the initial core temperature upto 15^{o} to 18^{o} C before transferring it to fermentation tanks. The reduced temperature helps to prevent oxidation and browning of musts/juice. The musts/juice is then transferred to fermentation tanks by using volumetric pumps. The juice is then clarified by allowing it to stand for 24 - 48 hours at 5^{o} to 10^{o} C in the tanks. Enzymes are also added to accelerate the breakdown of cell wall/tissues and to improve the clarity of juice. The clarified grape juice is first chilled in a combination of stainless steel tanks and oak barrels where fermentation begins either spontaneously or it is induced by the addition of a starter culture of yeast (*Saccharomyces cerevisiae).*This process is called the first fermentation of wine. The ethanol formed during first fermentation assists the extraction of pigments from the skins. This process of colour development takes around 24 hours to three weeks depending on the colour of the final product required.

The skins are then removed and the partially fermented wine is transferred to a separate tank for complete fermentation. Fermentation is generally carried out in large stainless steel vessels at 10 -18^{o} C for about seven to eight weeks. The low temperature and slow rate fermentation favours to retain the volatile compounds. Fermentation stops naturally when all the fermentable sugars are converted to alcohol or when the alcoholic strength reaches the limit of tolerance of the strain of yeast involved.

Sometimes the wine becomes acidic due to higher concentration of L - malic acid which is produced by fermentation of musts from not fully ripened grapes. Malolactic fermentation primarily occurs in wines where acidity is less than 1g/litre tartaric acid and pH higher than 3.1. Since tartaric acid present in musts is stable to the actions of *Saccharomyces cerevisiae* strain of yeast, the microbial degradation of malic acid is done by using another strain of yeast called *Saccharomyces pombe.* Malolactic fermentation also imparts a buttery flavour to the wine and the process is used for sparkling wines.

THE DETAILED FLOW CHART OF RED AND WHITE WINE PROCESSING IS AS UNDER



Once fermentation process is over, the process of racking is carried out in which the wines are allowed to still until most of the yeast cell and fine suspended materials settle out. The racking process is undertaken for about 22 - 25 days. Further, about 8 – 10 rackings are recommended / required to make a crystal clear wine without any impurities and sedimentations or tartaric acids. The wine is then filtered without disturbing the sediments or the yeast. The wines may be then kept in the oak barrels for several months to years for aging to develop a particular aroma and flavours. No air is allowed to enter the barrels during this aging period. The process of barrel aging gives the wine a complex flavour which comes from the tannin of oak wood. Once the mature wine is ready, it is stabilised through a cold treatment process at - 4 ^{o} C for about a week to remove all the tartaric acids present in the wine. The process is called Cold Stabilization, which prevents cloudiness and setting of crystal particles in the bottle.

It also helps to make a softer wine. After testing the stability of the wine, it is filtered to screen the rest of fine particles. The wine undergoes a series of filtration process to make it crystal clear and shiny. The various types of machinery used in filtration process are Plate & Frame Filter, Cartridge Filter and Kisulger Filter. Once the final filtration is over, the wine is packed in bottles and stored in cool and dry places where the exposure to sunlight is minimum. While storing the wine, the bottles are always kept horizontally so that corks shall always remain moist to prevent them from drying and any air slipping inside the bottles.

Cider - fermentation - filtration - storage

Cider is an alcoholic drink made from crushed and then fermented apples. Cider is known as *hard cider* in the United States and parts of Canada, a term that differentiates alcoholic cider from a type of apple juice known as *cider*.Cider generally has a stronger alcoholic content than beer, usually over 5%, and golden yellow or cloudy appearance. To produce cider, apples are washed, then grated or mashed, then pressed (usually in a stone mill or hydraulic press) then fermented in oak vats using natural or added yeasts.

Cider is very popular in the United Kingdom, especially in South West England, when compared with other countries, and the UK has the highest per capita consumption as well as the largest cider producing companies in the world [1] including Bulmers, the largest [2]. The drink is also popular and traditional in Brittany and Normandy (France), in Ireland and northern Spain. The Netherlands and Germany also produce cider. The drink is making a resurgence in both Europe

and the United States [3].Eating apples are far from ideal for cidermaking, as they are low in tannins. Most makers use cider apples, the cultivars developed specifically for cidermaking, of which there are many hundreds.

Types of cider

Cider comes in a variety of tastes, from sweet to dry, although flavour differs enormously within these descriptions. The appearance of cider ranges from very dark, cloudy and sludgy through to very crisp, clean and golden yellow, and with the most processed, almost entirely clear. The varying colours and appearances are generally as a result of how much of the apple material is removed between pressing and fermentation.Modern, mass-produced ciders are generally heavily processed and resemble sparkling wine in appearance. More traditional brands tend to be darker and cloudier, as less of the apple is filtered out.

They are often stronger than processed varieties."White cider" is made by processing cider after the traditional brewing process is complete, resulting in a nearly white product. This processing allows the manufacturer to produce strong (typically 7-8% ABV) cider cheaply, quickly, and on an industrial scale, often from poor quality raw materials.More in depth descriptions of some of the various types of cider are available under the country headings below.

Cider production

Most cider is made industrially nowadays, although traditional methods still survive. In this picture the layers of pomace are wrapped in canvas.Once the apples are gathered from trees in orchards they are "scratted" (ground down) into what is called "pomace" or "pommage". Historically this was done using pressing stones with circular troughs, or by a cider mill. Cider mills were traditionally driven by the hand, water-mill, or horse-power. In modern times they are likely to be powered by electricity. The pulp is then transferred to the cider "press", where the pommage is pressed and formed by pressure into a kind of cake, which is called the "cheese".Traditionally the method for squeezing the juice from the cheese involves placing clear, sweet straw or hair cloths between the layers of pomace. This will usually alternate with slatted ash-wood racks, until there is a pile of ten or twelve layers. It is important to minimise the time that the pomace is exposed to air and to minimise oxidation. The cheese needs to be constructed evenly, or the whole pile slithers onto the floor.This pile is then subjected to different degrees of pressure in succession, until all the 'must' or juice is squeezed from the pomage. This juice, after being strained in a coarse hair-sieve, is then put into either open vats or

closed casks. The pressed pulp is given to farm animals as winter feed, or discarded, or used to make liqueurs.

Fermentation

Fermentation is best effected at a temperature of 4–16 °C (40–60°F). This is low for most kinds of fermentation, but works for cider as it leads to slower fermentation with less loss of delicate aromas. Shortly before the fermentation consumes all the sugar, the liquor is "racked" into new vats. This leaves dead yeast cells and other undesirable material at the bottom of the old vat. At this point it becomes important to exclude airborne acetic bacteria, so care is taken to fill the vat completely, and the fermenting of the remaining available sugar generates a small amount of carbon dioxide that helps to prevent air seeping in.

This also creates a certain amount of sparkle, and sometimes extra sugar is added at this stage for this purpose and also to raise the alcohol level. Racking is sometimes repeated if the liquor remains too cloudy.The cider is ready to drink at this point, though more often it is matured in the vats for up to two or three years.[

Blending and bottling

For larger-scale cider production, ciders from vats produced from different varieties of apple may be blended to accord with market taste. If the cider is to be bottled, usually some extra sugar is added for sparkle. Higher quality ciders can be made using the champagne method, but this is expensive in time and money and requires special corks, bottles, and other equipment

Fermented cereal, pulse and cassava based products , malted, germinated, high fructose, malto dextrose products.

Indian diet primarily consists of cereals and pulses. According to a recent finding, proteins and protective foods are not available to the average Indian. If every one is given a balance diet there will be a shortage of 27 per cent of food grains and 50 per cent of pulses, besides huge deficiency of fruits, vegetables, oil, fats, milk, etc.

In India, the common cereals and millets consumed are rice, wheat, maize, sorghum, ragi and pearl millet. The grains are rich sources of energy, calcium, iron and B group vitamins. These millets along with maize and sorghum are considered as coarse grains and constitute the food of the weaker sections of dry land regions in India.

The small millets like varagu, thenai, samai, panai varagu and kudhirai valli and legumes like cow pea, horse gram and soya bean also coming under underutilized millets and legumes Combination of these two will increase the nutritive value. But some of the anti nutritional factors like trypsin inhibitor, heamoagglutenin, tannin, alkaloids present in the grains which can be eliminated by soaking; germinating, roasting, etc.Combination of these is known as malting. A small amount of any whole cereal grain is steeped overnight in 2-3 times its volume of water. The excess water is drained, allowed for germinaton for 24-48 hours, dried for 5-7 hours, lightly roasted to remove surface moisture and sprouts are removed by aberration and grains are milled. During malting vitamins like vitamin B complex and vitamin C are increased and complex form of carbohydrates and proteins are converted in to more digestible forms Malted foods are also known as Amylase Rich Food (ARF). Just tiny or catalytic amounts of any germinated cereal flour can instantly reduce dietary bulk.

These remarkable properties make it possible to offer the highly nutritious weaning food and health mixes. The advantages of malting are increase in nutritive value and bioavailability of proteins, easy to de husk the grains and to digest and decrease the cooking time.

Corn Syrup

The development of the various types of corn syrups, maltodextrins, and high-fructose corn syrup from corn starch sources could be called one of the greatest achievements in the sugar industry. Corn starch can be hydrolyzed into glucose relatively easily, but it was not until the 1970s that it became a commercially major product bringing about changes in the food industry. The starch is processed and refined from the kernels of corn by using a series of steeping (swelling the kernel), separation, and grinding processes to separate the starch from the other parts of the kernel which is used for animal feed.

The starch is hydrolyzed using acid, acid-enzyme, or enzyme-enzyme catalyzed processes. The first enzyme is generally a thermally stable alpha amylase which produces about 10-20 % glucose. Further treatment with the enzyme glucoamylase yields 93-96% glucose. The final Corn Syrup (glucose syrup) products include: dried corn syrup, maltodextrin, and dextrose (glucose).

Glucose Isomerase:

With the development of glucoamylase in the 1940s and 1950s it became a straightforward matter to produce high percent glucose syrups.

However, these have shortcomings as used in the sweetener industry. D-glucose has only about 70% of the sweetness of sucrose, on a weight basis, and is comparatively insoluble. Fructose is 30% sweeter than sucrose, on a weight basis, and twice as soluble as glucose at low temperatures so a 50% conversion of glucose to fructose overcomes both problems giving a stable syrup that is as sweet as a sucrose solution of the same concentration.

One of the triumphs of enzyme technology so far has been the development of 'glucose isomerase', which in turn led to the commercialization of high fructose corn syrups. Now it is known that several types of bacteria, can produce such glucose isomerases. They are resistant to thermal denaturation and will act at very high substrate concentrations, which have the additional benefit of substantially stabilizing the enzymes at higher operational temperatures.

The vast majority of glucose isomerases are retained within the cells that produce them but need not be separated and purified before use. All glucose isomerases are used in immobilised forms. Although differerent immobilisation methods have been used for enzymes from differerent organisms, the principles of use are very similar. The corn syrup is then converted to fructose in a batch process to make 42% fructose syrup.

For many purposes a 42% fructose syrup is perfectly satisfactory for use but it does not match the exacting criteria of the quality soft drink manufacturers as a replacement for sucrose in acidic soft drinks. For use in the better colas, 55% fructose is required. This is produced by using vast chromatographic columns of zeolites or the calcium salts of cation exchange resins to adsorb and separate the fructose from the other components.High fructose corn syrups are classified according to the fructose content (i.e. 42%, 55%, 90%).

High fructose corn syrup (HFCS) is a newer and sweeter form of corn syrup. Like ordinary corn syrup, the high fructose variety is made from corn starch using enzymes. The production process of HFCS was developed by Japanese researchers in the 1970s. HFCS was rapidly introduced in many processed foods and soda drinks in the US over the period of about 1975–1985, and usage continues to increase as sugar use decreases at a nearly one to one level (Bray, 2004 & U.S. Department of Agriculture, Economic Research Service, Sugar and Sweetener Yearbook series, Tables 50–52.). There are three main reasons for this switch; first is cost, as HFCS is a bit cheaper due to corn subsidies and import sugar tariffs.

The second reason is that it is a liquid which is easier to blend and transport. The third is that a product made with HFCS has a much longer shelf life. (White JS. 1992. Fructose syrup: production, properties and applications, in FW Schenck & RE Hebeda, eds, Starch Hydrolysis Products – Worldwide Technology, Production, and Applications. VCH Publishers, Inc. 177-200) By increasing the fructose content of corn syrup (glucose) through enzymatic processing, the syrup is more comparable to table sugar (sucrose). This makes it useful to manufacturers as a possible substitute for sugar in soft drinks and other processed foods. Common commercial grades of high fructose corn syrup include fructose contents of 42%, 55%, or 90%. The 55% grade is most commonly used in soft drinks and equivalent to caster sugar. Unlike sucrose, HFCS consists of a mixture of glucose and fructose, which doesn't require an enzymatic step to break it down before absorption in the intestine.

Production

High-fructose corn syrup (HFCS) is produced by processing corn starch to yield glucose, and then processing the glucose to produce a syrup with a higher percentage of fructose. First, cornstarch is treated with alpha-amylase to produce shorter chains of sugars called oligosaccharides. Then, an enzyme called glucoamylase breaks the sugar chains down even further to yield the simple sugar glucose.

The third enzyme, glucose isomerase, converts glucose to a mixture of about 42% fructose and 50–52% glucose with some other sugars mixed in. While alpha-amylase and glucoamylase are added directly to the slurry, glucose-isomerase is packed into columns and the sugar mixture is then passed over it. This 42–43% fructose glucose mixture is then subjected to a liquid chromatography step where the fructose is enriched to approximately 90%. The 90% fructose is then back-blended with 42% fructose to achieve a 55% fructose final product. Numerous ion-exchange and evaporation steps are also part of the overall process.

UNIT – V

Bioremediation

Bioremediation is the biological treatment and removal of pollution from the environment. The Principal organizer in bioremediation are bacteria and fungi that have the ability to degrade hydrocarbons such as oil and coal, tar, and Xenobiotics such as pesticides. Although metals cannot be degraded they can be accumulated by microorganism and there removed from the environment. As bioremediation uses mixed populations of microorganisms the dynamics of such populations are complex.

Removal or reduction of pollution from current process

Biotechnology offers some of the most environmentally friendly solutions. Examples are the removal of metals and radio nucleotides from waste accumulation of metals.

Prevention of pollution (Clean technology)

Biotechnology can contribute towards the cleaner production of existing products. One example is the microbial removal of sulphur compounds from coal prior to combustion. Another is the use of fungi to pretreat logs before pulp and paper production, which reduces the use of energy and bleaching materials.

Luminescent

MICROTOX ™, BioTox™, Tox Alret ® and LUMISTox® are systems that use the reduction is light emission by luminous bacteria such as Photobacterium tisceri and photobacterium phosphoseum as a measurement of the toxicity of a compound over a 15-min exposure period.

Bioremediation strategies.

- Use the indigenous microbial population

- Encourage the indigenous population

- Bioargumentation is the addition of selected organism to contaminated sites in order to supplements the indigenous microbial population and speed up degradation.

- Genetically manipulated organism

The creation of superbug by genetic manipulation bas been considered for same time. A multiplasmid – containing pseudomonas strain has been produced which is capable of oxidizing aliphatic, aromatic, terpenic and hydrocarbon. Another multiplasmid organism, Pseudomonas putida, has been produced which can degrade both lighter alkanes and aromatics (Venosa and Zhu, 2003).

Members of the genus Pseudomonas (a soil microorganism) are the most predominant microorganism that degrade Xenobiotics. Different strains of Pseudomonas, that are capable of detoxifying more than 100 organic compounds, have been identified. About 40-50 microbial strains of microorganisms, capable of degrading Xenobiotics have been isolated. Besides Pseudomonas, other good examples are Mycobacterium, *Alcaligenes* and *Nocarida*.

Genetic Engineering for more efficient Bioremediations.

Although several microorganisms that can degrade a large number of Xenobiotics have been identified, there are many limitations.

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

-No single microorganism can degrade all the Xenobiotics present in the environment.

-The growth of the microorganism may be inhibited by the Xenobiotics.

Some attempt have been made in recent years to create genetically engineered microorganisms (GEMs) to enhance bioremediation.

Genetic manipulation by transfer of plasmids

The majority of the genes responsible for the synthesis of biodegradative enzymes are located on the plasmids. It is therefore logical to think of genetic manipulations of plasmids.

A selected list of Xenobiotics and the plasmids containing genes for degradation.

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

Genetic manipulation by transfer and 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 region of DNA, recombination occurs between them, resulting in the formation of a larger fused plasmid. In case of plasmids which do not possess homologous regions of DNA, they can coexist in the bacterium.

The first successful development of a new strain of bacterium (Pseudomonas) by manipulation of plasmid transfer was done by Chakrabarty and his co-workers in 1970s. The new bacterium called superbug, became the first genetically engineered microorganisms to be patented (US patent in 1981).

Creation of superbug of transfer of plasmids

Superbug is a bacterial strain and pseudomonas that can degrade camphor, octane, Xylene and naphthalene.

Genetically Engineered Microorganism in Bioremediation.

A selected list of GEMs with a potential for the degradation of xenobiotic is given. Almost all these GEMs have been created by transferring plasmids.

GEMs	Xenobiotics
Pseudomanas diminuta	Parathion
P.Olevorans	Alkane
P.Putida	Mono + dicholoro aromatic compounds
Alcaligens SP	2, 4 – Dichlorophenozy acetic acid
Acinetobactes SP	4 – Chlorobenzene

Because of the risks involved in the use of GEMs. So far no GEM have been allowed to enter me environmental fields. Thus, the use of GEMs has been confined to the laboratories and fully controlled processes of biodegradation (usually employing bioreactors). The use of GEM in environment is possible only if the associated risks of each GEM is thoroughly evaluated and fully assured of its biosafety.

Phytoremediation

Phytoremediation is the use of plants for the removal of contaminants and methods from soil and water or to render them harmless. The plants that accumulate high concentrate of metals are known as hyperaccumulators and can accumulate 50 – 100 times more metal than normal plants. Examples of hyper accumulators are *Thlaspi caerulescens* and *Carduminopsis halleri*, which accumulate Zinc and Cadmium. *Alyssum lesbiacum* accumulates nickel and the fern *Pteris Vittata* accumulates arsenic. One of the most studies mechanism for metal sequestration is by the peptides. It has been shown that the metallothioneins and phytochelatins are stimulated by exposure to metals.In Thlaspi, zinc transports gene was isolated.. This results is very high movement of zinc transport from the soil and movement of this metal to the leaves.

The most common types of phytoremediation are:

-Rhizotilteration: Uptake of contaminants in roots (cleaning soil water)

-**Phytoextraction:** Taking contaminants from soil and storing in root or the shoots.

-**Phytotransformation:** involves degradation of contaminants through plant metabolism and can take pollutants from soil or water.

-Phytostabilization : Using plants to reduce the mobility of contaminants in the soil, locking them in place

-Phytovolatilization: Refers to the uptake and transpiration of contaminates (released into the atmosphere where it evaporated or vaporizes.

Phytoremediations future lines with the transgenic.

When mercury in dumped, microbes present in soil and water convert it two methyl mercury, which accumulates into food chain causes neurological problem. Meagher and Coworker isolated a bacterial enzyme, called mercuric ion reductase, that converts the metal into its least toxic form. When transferred to Arabidopsis it allowed the plant to grow in soil heavily contaminated with mercury and release it into the air, using the afore mentioned process of phytovolatilization. The contamination of found water by arsenic in Bangladesh is the largest poisoning of a population in history. Richard Meagher and Coworker, University of Georgia, USA interested two unrelated *E. coli* genes into Arabidopsis, allowing it to remove arsenic from the soil and transport it to the plant leaves. The transgenic plants accumulated seventeen times as much fresh shot weight and two or three times as much arsenic per gram of tissue as non-transgenic plants.

Poplar trees have already been used to remove chlorinated solvents and nitrates from groundwater and heavy metals from soil.

Bacteria: One bacterium they commonly use is Geobacter. It stops uranium in its tracks so it doesn't make it into place for over ten thousand years.

WASTEWATER TREATMENT AND DISPOSAL

Raw or untreated sewage is mostly pure water. Infact, sanitary waste water comprises about 99.9 per cent water and only about 0.1 per cent impurities. In other words, if a 1-L (1-kg) sample of wastewater is allowed to evaporate, only about 1g, or 1000 mg of solids will remain behind.

In contrast to this, sea water is only about 96.5 per cent pure water; it contains about 35,000 mg/L, or 3.5 per cent dissolved impurities.

Although sea water contains more impurities than does sanitary sewage, we do not ordinarily consider seawater to be polluted. The important distinction is not the total concentration, but the type of impurities. The impurities in seawater are mostly inorganic salts, but sewage contains biodegradable organic material, and it is very likely to contain pathogenic microorganisms as well.

Actually, sewage can contain so many different substances, both suspended and dissolved, that it is impractical to attempt to identify each specific substance or microorganisms. The total amount of organic materials is related to the strength of the sewage. This is measured by the biochemical oxygen demand, or BOD. Another important measure or parameter related to the strength of the sewage is the total amount of suspended solids, or TSS. On the average, untreated domestic sanitary sewage has a BOD of about 200 mg/L and a TSS of about 240 mg/L. Industrial wastewater may have BOD and TSS values much higher than those for sanitary sewage; its composition is source dependent.

Another group of impurities that is typically of major significance in waste water is the plant nutrients. Specifically, these are compounds of nitrogen and phosphorous. On the average, raw sanitary sewage contains about 35 mg/ L of N and 10 mg / L of P.

Finally, the amount of pathogens in the waste water is expected to be proportional to the concentration of fecal coli form bacteria. The coli form concentration in raw sanitary sewage is roughly 1 billion per liter. Coli form concentration, as well as BOD, TSS, and concentrations of N and P, are parameters of water quality.

Before discharging wastewater back into the environment and the natural hydrologic cycle, it is necessary to provide some degree of treatment in order to protect public health and environmental quality. The basic purposes of sewage treatment are to destroy pathogenic microorganisms and to remove most suspended and dissolved biodegradable organic materials. Sometimes it is also necessary to remove the plant nutrients – nitrogen and phosphorous. Disinfection, usually with chlorine, serves to destroy most pathogens and helps to prevent the transmission of communicable disease. The removal of organics (BOD) and nutrients helps to protect the quality of aquatic eco-systems.

These treatment methods are grouped into three general categories: primary treatment, secondary or biological treatment and tertiary or advanced treatment.

Screening		
Grit removal	Screening / grit disposal	
Sedimentation	Sludge	
Secondary treatment	Sludge	Sludge treatment
Tertiary treatment	Sludge	
Disinfection		Sludge disposal
	Treated wastewater effluent	

PRELIMINARY AND PRIMARY TREATMENT

Untreated or raw wastewater usually flows by gravity from an interceptor or trunk sewer into the head works of a treatment facility; sometimes wastewater may be pumped to the treatment plant in a force main. The head works of a treatment plant include a flow measurement device such as a Par shall flume and mechanical systems that provide preliminary treatment. Preliminary treatment systems typically include screens, comminutors, and grit chambers.

The first treatment process for raw wastewater is coarse screening. Bar screens (or racks), as they are called, are made of long, narrow metal bars spaced about 25 mm (1 in.) apart. They retain floating debris, such as wood rags, or other bulky objects, that could clog pipes or damage mechanical equipment in the rest of the plant.

In some treatment plants, a mechanical cutting or shredding device, called a comminutor, is installed just after the coarse screens. The comminutor shreds and chops solids or rags that passed through the bar screen. The shredded material is removed from the waste water by sedimentation or flotation later in the treatment plant.

Grid removal

A portion of the suspended solids in raw sewage consists of gritty material, such as sand, coffee grounds, eggshells, and other relatively inert material. In cities with combined sewer systems, sand and silt may be carried in the sewage. Suspended grit can cause excessive wear and tear on pumps and other equipment in the plant. Most of it is non biodegradable and will accumulate in treatment tanks. For these reasons, a grit removal process is usually used after screening and / or comminuting.

Primary sedimentation (Settling)

After preliminary treatment by screening, comminuting, and grit removal, the wastewater still contains suspended organic solids that can be removed by plain sedimentation. Settling tanks that receive sewage after grit removal are called primary clarifiers. The combination of preliminary screening and gravity settling is called primary treatment. Chemicals may sometimes be added to the primary clarifiers to promote the removal of very small (or colloidal) particles. Primary treatment usually can remove up to 60 per cent of the suspended solids and about 35 per cent of the BOD from wastewater, but this relatively low level of treatment is no longer adequate.

In almost all cases, primary treatment must be followed by secondary treatment processes; tertiary treatment may also be required to protect sensitive bodies of water that receive the treated effluent.

SECONDARY (BIOLOGICAL) TREATMENT

Primary treatment processes remove only those pollutants that will either float or settle out by gravity, but about half of the raw pollutant load still remains in the primary effluent. The purpose of secondary treatment is to remove the suspended solids that did not settle out in the primary tanks and the dissolved BOD that is unaffected by physical treatment. Secondary treatment is generally considered to meet 85 per cent BOD and TSS removal efficiency and represents the minimum degree of treatment required in most cases.

Biological treatment of sewage involves the use of microorganisms. The microbes, including bacteria and protozoa, consume the organic pollutants as food.

They metabolize the biodegradable organics, converting them into carbon dioxide, water and energy for their growth and reproduction. A biological sewage treatment system must provide the microorganisms with a comfortable home. In effect, the treatment plant allows the microbes to stabilize the organic pollutants in a controlled, artificial environment of steel and concrete, rather than in a stream or lake. This helps to protect the dissolved oxygen balance of the natural aquatic environment.

To keep the microbes happy and productive in their task of wastewater treatment, they must be provided with enough oxygen, adequate contact with the organic material in the sewage, suitable temperatures, and other favourable conditions. The design and operation of a secondary treatment plant is accomplished with these factors in mind.

Two of the most common biological treatment systems are the trickling filter and the activated sludge process. The trickling filter is a type of fixed growth system. The microbes remain fixed or attached to a surface while the wastewater flows over that surface to provide contact with the organics. Activated sludge is characterized as a suspended – growth system, because the microbes are thoroughly mixed and suspended in the waste water rather than attached to a particular surface.

Trickling filters

A trickling filter consists basically of a layer or bed of crushed rock about 2m (6ft) deep. It is usually circular in shape and may be built as large as 60 m (200 ft) in diameter. Trickling filters are always preceded by primary treatment to remove coarse and settleable solids. The primary effluent is sprayed over the surface of the crushed stone bed and trickles downward through the bed to an under drain system.

A rotary distributor arm with nozzles located along its length is usually used to spray the sewage, although sometimes fixed nozzles are used. The rotary distributor arm is mounted on a center column in the trickling filter; it is driven around by the reaction force or jet action of the waste water that flows through the nozzles.

The under drain system serves to collect and carry away the wastewater from the bottom of the bed and to permit air circulation upward through the stones. As long as topography permits, the sewage flows from the primary tank to the trickling filter by the force of gravity, rather than by pumping.

As the primary effluent trickles downward through the bed of stones, a biological slime of microbes develops on the surfaces of the rocks. The continuing flow of the wastewater over these fixed biological growths provides the needed contact between the microbes and the organics. The microbes in the thin slime layer absorb the dissolved organics, thus removing oxygen – demanding substances from the waste – water. Air circulating through the void spaces in the bed of stones provides the needed oxygen for stabilization of the organics by the microbes.

Note, however, that the trickling filter is not really a filter at all, in the true sense of the word. The stones are usually about 75 mm (3 in.) in size, much too large to filter out suspended solids. And, by definition, filters have no effect on dissolved solids. The stones in a trickling filter only serve to provide a large amount of surface area for the biological growths, and the large voids allow ample air circulation.

The trickling filter effluent is collected in the under drain system and then conveyed to a sedimentation tank called a secondary clarifier. The secondary clarifier, or final clarifier as it is sometimes called, is similar in most respects to the primary clarifier, although there are differences in detention time, over flow rate, and other details.

To maintain a relatively uniform flow rate thorough the trickling filter and to keep the distributor arm rotating even during periods of low sewage flow, some of the waste water may be recirculated. In other words, a portion of the effluent is pumped back to the trickling filter inlet so that it will pass through the bed of stones more than once.

Recirculation can also serve to improve the pollutant removal efficiency; it allows the microbes to remove organics that flowed by them during the previous pass through the bed.

Activated sludge treatment

The basic components of an activated sludge sewage treatment system include an aeration tank a secondary settling basin or clarifier. Primary effluent is mixed with settled solids that are recycled from the secondary clarifier and then introduced into the aeration tank. Compressed air is injected continuously into the mixture through porous diffusers located at the bottom of the tank along one side.

In the aeration tank, microorganisms consume the dissolved organic pollutants as food. The microbes absorb and aerobically decompose the organics, using oxygen provided in the compressed air; water, carbon dioxide and other stable compounds are formed. In addition to providing oxygen, the compressed air thoroughly mixes the microbes and wastewater together as it rapidly bubbles up to the surface from the diffusers. Sometimes mechanical propeller like mixers, located at the liquid surface, are used instead of compressed air and diffusers. The churning action of the propeller blades mixes air with the wastewater and keeps the contents of the tank in a uniform suspension.

The aerobic microorganisms in the tank grow and multiply, forming an active suspension of biological solids called activated sludge. The combination of the activated sludge and waste water in the aeration tank is called the mixed liquor. In the basic or conventional activated sludge treatment system, a tank detention time of about 6h is required for thorough stabilization of most of the organics in the mixed liquor.

After about 6h of aeration, the mixed liquor flows to the secondary or final clarifier, in which the activated sludge solids settle out by gravity. The clarified water near the surface, called the supernatant, is discharged over an effluent weir; the settled sludge is pumped out from a sludge hopper at the bottom of the tank. Recycling a portion of the sludge back to the inlet of the aeration tank is an essential characteristic of this treatment process. The settled sludge is in an active state. In other words, the microbes are well acclimated to the wastewater and, given the opportunity, will readily absorb and decompose more organics by their metabolism.

By pumping about 30 per cent of the wastewater flow from the bottom of the clarifier back to the head of the aeration tank, the activated sludge process can be maintained continuously.

When mixed with the primary effluent, the hungry microbes quickly begin to absorb and metabolize the fresh food in the form of BOD causing organics. Since the microbes multiply and increase greatly in numbers, it is not possible to recycle or return all the sludge to the aeration tank. The excess sludge, called waste activated sludge, must eventually be treated and disposed of (along with sludge from the primary tanks).

Tertiary (Advanced) Treatment

Secondary treatment can remove between 85 and 95 per cent of the BOD and TSS in raw sanitary sewage. Generally, this leaves 30 mg / L or less of BOD and TSS in the secondary effluent. But sometimes this level of sewage treatment is not sufficient to protect the aquatic environment.

Another limitation of secondary treatment is that it does not significantly reduce the effluent concentrations of nitrogen and phosphorous in the sewage. Nitrogen and phosphorous are important plant nutrients. If they are discharged into a lake, algal blooms and accelerated lake aging or cultural eutrophication may be the result. Also, the nitrogen in the sewage effluent may be present mostly in the form of ammonia compounds. These compounds are toxic to fish if the concentrations are high enough. Yet another problem with the ammonia is that it exerts a nitrogenous oxygen demand in the receiving water as it is converted to nitrates. This process is called nitrification.

When pollutant removal greater than that provided by secondary treatment is required, either to further reduce the BOD or TSS concentrations in the effluent or to remove plant nutrients, additional or advanced treatment steps are required. This is also called tertiary treatment, because many of the additional processes follow the primary and secondary processes in sequence.

Tertiary treatment of sewage can remove more than 99 per cent of the pollutants from raw sewage and can produce an effluent of almost drinking water quality.

TECHNOLOGIES IN SOLID WASTE MANAGEMENT

Solid waste management is a difficult process because it involves many disciplines.These include, technologies associated with the control of generation, storage, collection, transfer and transportation, processing, marketing, incineration and disposal of solid wastes. All of these processes have to be carried out with in existing legal and social guidelines that protect the public health and environment and are aesthetically acceptable. They must be responsive to public attitudes and the disciplines included in the disposal process, include administrative, financial, legal, architectural, planning and engineering functions. For successful integrated solid waste management plant, it is necessary that all these disciplines communicate and interact with each other in a positive interdisciplinary relationship .The various techniques employed in solid waste management include,

1) Composting
2) Sanitary land filling (Controlled tipping)
3) Thermal process (Incineration and pyrolysis)
4) Recycling and reuse

COMPOSTING

It is being increasingly realized that composting is an environment friendly process to convert wide variety of wastes into valuable agricultural inputs. This process minimizes the environmental problems. Composts are excellent source of humus and plant nutrients, the application of which improves soil bio physical properties and organic matter status of the soil. Composting can be defined as the biological conversion of organic wastes into an amorphous dark brown to black colloidal humus like substance under conditions of optimum temperature, moisture and aeration. Nutrient content of compost depends largely on the nutrient content of the wastes. Composting is a process in which the organic portion of solid waste is allowed to decompose under carefully controlled conditions. It is a biological rather than a chemical or mechanical process; decomposition and transformation of the waste material are accomplished by the action of bacteria, fungi, and other microorganisms.

With proper control of moisture, temperature, and aeration, a composting plant can reduce the volume of raw organic material by as much as 50 per cent. In addition, composting can stabilize the waste and produce an end product that may be recycled for beneficial use.

The end product is called compost or humus. It resembles potting soil in texture and earthy odor, and it may be used as a soil conditioner or mulch.

A complete municipal solid waste (MSW) composting operation includes sorting and separating, shredding and pulverizing, digestion, product upgrading, and finally marketing. Sorting and separation operations are required to isolate organic, decomposable waste materials from the plastic, glass, metal, and other non biodegradable substances. Solid waste sorting and separation methods are a key part of MSW recycling operations.

Shredding and pulverizing serve to reduce the size of the individual pieces of the organic waste, resulting in a relatively uniform mass of material. This facilitates handling, moisture control, and aeration of the decomposing waste. Size reduction also helps optimize bacterial activity and increases the rate of decomposition. After size reduction, the wastes are ready for the actual composting or digestion step. Digestion may take place in open windrow or in an enclosed mechanical facility. A windrow is a long, low pile of the prepared organic waste, usually about 3m (10 ft) wide at the base and about 2 m (6 ft) high. Most windrows are conical in cross section and about 50 m (150 ft) in length. The composting waste is aerated by periodically turning each windrow. Turning frequency varies with moisture content and other factors. When moisture content is maintained at about 50 per cent, windrows are turned two or three times a week and in some cases daily. Generally, open – field windrow composting takes about 5 weeks for digestion orstabilization of the waste material. An additional 3 weeks may sometimes be required toensure complete stabilization. Temperatures in an aerobic compost windrow may reach 5^{o}C (150^{o}F) because of the natural metabolic action of thermophilic microbes that thrive t such elevated temperatures. The relatively high temperatures destroy most of the pathogenic or disease-causing organisms that may be present in the waste. Open-field windrow composting requires relatively large land areas. To reduce land requirements, various types of enclosed mechanical systems can be used in lieu of the open-field method. A variety of mechanical type compost systems are available.

Oxygen is supplied to the waste material by forced aeration, stirring, or tumbling. In addition to reducing land requirements, enclosed mechanical compost facilities can reduce the time required for stabilization from about 5 weeks to about 1 week.

Composting is the aerobic, thermophilic degradation of organic matter present in the refuse by microbes, predominantly by fungi and actinomycetes, which are favoured by semi moist condition that prevail in the process. The control parameters for optimum composting include, temperature (40^{o}C), moisture (40.7%), pH (4.5 – 9.5), nutrients (C:N ratio 40:1); C:P ratio (100:1), air (0.5 – 0.8 m / d / kg volatile solid) and particle size (6-25 mm). The digestion of the waste is carried out naturally in an outside decomposition area in windrows (for five weeks) or in mechanized composting plants (for 4 to 6 days). In natural system, the garbage is mixed with nutrient source (sewage sludge / animal manure) and a filler (wood chips) to provide entry of air. The mixture is turned over twice a week and the process is completed in 4-6 weeks. The darkening of refuse, fall in temperature and a musty odour indicate completion of the process. Before the stabilized compost or humus can be sold for use as a mulch or soil conditioner, it must be processed further to upgrade or improve its quality and appearance. This includes drying, screening, and granulating or pelletizing. Sometimes, the compost is placed in bags, although bulk sale is more efficient and economical.Compost can increase the organic and nutrient content of soil and improve its texture and ability to retain moisture.

Co-Composting

An interesting example of integrated waste management is co-composting of municipal solid waste and sewage sludge. Sewage sludge adds nitrogen, phosphorous, and other elements that enrich the solid waste and help the composting process. The sludge is first dewatered so that it does not add too much moisture to the compost pile. The dewatered sludge and organic portion of MSW must be thoroughly mixed. At a time when ocean disposal of sludge has been banned and sludge incinerators meet with much public opposition, co-composting may offer an increasingly viable technique for processing both sludge and MSW organics prior to final disposal.

Vermicomposting

The key role of earthworms in improving the soil fertility is well known for a longer period. Earthworms feed on any organic wastes, consume three to five times their body weight and after using 5 to 10 per cent of the organic wastes for their growth, excrete the mucus coated undigested matter as worm casts.

Worm casts consist of organic matter that has undergone physical and chemical breakdown through the activity of the muscular gizzard, that grinds the material to a particle size of 1-2 micron. The nutrients present in the worm casts are readily soluble in water for the uptake of plants. Vermicastings are rich sources of macro and micronutrients, vitamins, enzymes, antibiotics, growth hormones and immobilized micro flora.

Vermicompost refers to organic manure produced by earthworms. It is a mixture of worm castings, including humus, live earthworms, their cocoons and other micro organisms. Vermicomposting is an appropriate method for disposal of non-toxic solid and liquid organic wastes. It helps in cost effective and efficient recycling of animal wastes (Poultry droppings, horse, piggery excreta and cattle dung), agricultural residues and industrial wastes using low energy.

Types of earthworms

Several types of earthworms are found in our soils. Earthworms can be divided into the following two categories:

1. Epigeic – the surface living worms
2. Endogeic – the burrowing worms

Epigeic: These worms are found on the surface and are reddish brown in colour. They do not process the soil but are efficient in composting of organic wastes. They enhance the rate of organic manure production through biodegradation or mineralization.

eg. Lampito mauritii, Octochaetona serrata, Perionyx excavatus

Endogeic: These species burrow and mix the soil, from different horizons in the profile. They ingest organic and mineral fraction of soil, thus promoting the formation of organo mineral complexes. Organo – mineral crumbs are brought from deeper parts of the soil profile to the surface. Different species of earthworms show specificity to soil types, moisture content and temperature.

Method of vermicomposting

Selection of earthworm: Earthworm that is native to the local soil may be used

Size of pit: Any convenient dimension such as 2m x 1m x 1m may be prepared

Preparation of vermibed: A layer, 15-20 cm thick of good loamy soil above a thin layer of (5 cms) broken bricks and sand should be made. Inoculation of earthworms: About one hundred earthworms are introduced as an optimum inoculating density into a compost pit of about 2m x 1m x 1m, provided with vermibed

Organic layering: It is done on the vermibed with fresh cattle dung. The compost pit is then layered to about 5 cm with dry leaves or hay or organic wastes. Moisture content of the pit is maintained by the addition of water. Wet organic layering: It is done after four weeks with moist green organic waste, which can be spread over it to a thickness of 5 cm. This practice can be repeated every 4 days. Mixing of wastes periodically without disturbing the vermibed ensures proper vermicomposting. Wet layering with organic wastes can be repeated till the compost pit is nearly full.

Harvesting of compost: At maturation (after 120 days), the moisture content is brought down, by stopping the addition of water. This ensures drying of compost and migration of worms in to the vermibed. The mature compost, a fine loose granular mass (about 1500 kg), is removed from the pit, sieved, dried and packed. Matured vermicompost is rich in nutrient and recommended @ 50 t ha^{-1}.

Vermibed for the preparation of vermicompost

	Jute cloth / net
4th layer - 15-20 cm	Kitchen wastes, garden wastes, mixed with cow dung
3rd layer -10 cm	100 Earthworms, loamy soil
2nd layer -15 to 20 cm	Coarse sand
1st layer – 5cm	Broken bricks / pebbles

Nutrient status of vermicompost prepared by *Perionyx excavatus*

Macronutrients	
Total nitrogen %	0.66
Total $P_2O_{5\%}$	1.93
Total $K_2O\%$	0.42
Micro nutrients	
Fe (ppm)	19.8
Zn (ppm)	0.90
Mn (ppm)	16.50
Cu (ppm)	2.30

General characters of vermicompost	
pH	7.00
EC dsm^{-1}	1.20
Organic carbon%	30.50
C: N Ratio	

Sanitary land filling (Controlled tripping)

Land filling is the most common and economic method of solid waste disposal. The indiscriminate land filling of solid waste in open dumps without adequate control and consideration of sanitation and public health as generally followed in India is dangerous. It results in water pollution, bad odour, fire and breeding of flies and rats.

It should be replaced by sanitary land filling or controlled tipping. The construction of sanitary land filling includes:

1) Deposition of solid waste in such a way to have a working force of minimum area.
2) Spreading and compaction of waste in thin layers
3) Covering of the waste with a layer of compacted cover soil daily.
4) Final cover of the entire construction with compacted earth layer of 1.0 m thick.

The solid wastes in sanitary land fill are degraded by soil microbes. In comparison with other biological treatment systems such as activated sludge and anaerobic digestion, the microbial degradation of solid waste proceeds at a slow rate.

Thermal process

Incineration

Incineration is a process of destruction of waste at high temperature. The combustible wastes are converted through controlled combustion to a residue, which contain no combustible matter. If land suitable for solid waste (SW) land filling operations is not available with in economic haul distances, then incineration is necessary. The solid waste is reduced in volume (80% - 90%) and height (98-99%).

Incinerator can accept toxic and industrial wastes of any size in solid or powdery form. The other special wastes include hospital wastes, putrifiable organic solids from slaughter houses.

Pyrolysis (Destructive distillation)

Pyrolysis is the process of conversion of biomass into solid, liquid and gaseous energy. Pyrolysis results in the chemical breakdown of organic carbon material into three basic components:

1) Gas phase containing mainly hydrogen, CO_2, CO and CH_4
2) tar or oil phase containing simple organic acids, methanol and acetone and
3) Pyrolysis does not cause pollution of the atmosphere and large quantities of potentially hazardous plastics could be treated.

There is no single prescription for an integrated waste management program that successfully works in every instance. Each situation must be analyzed on its own merit, an appropriate integrated waste management plan must be developed from hard data, and social attitudes and the legal frame work must be taken into account. The waste management disposal field is in a constant state of flux and appropriate solutions should be innovative, as well as technically and economically sound.

Sludge management

Suspended solids removed from wastewater during sedimentation and then concentrated for further treatment and disposal are called sludge or biosolids.

Even in fully aerobic waste treatment processes in which sludge is repeatedly recycled, most of the sludge must eventually be removed from the system.

The task of treating and disposing of this material is called sludge management.

Sludge characteristics

The composition and characteristics of sewage sludge vary widely. Since no two wastewaters are alike, the sludges produced will differ. Furthermore, sludge characteristics change considerably with time. Wastewater sludge typically contains organics (proteins, carbohydrates, fats oils), microbes (bacteria, viruses, protozoa), nutrients (phosphates and nitrates), and a variety of household and industrial chemicals. The higher the level of heavy metals and toxic compounds, the greater is the risk to humans and the environment. A key physical characteristic is the solids concentration, because this defines the volume of sludge that must be handled.

Sludge is treated prior to ultimate disposal for two basic reasons: volume reduction and stabilization of organics. Stabilized sludge does not have an offensive odor and can be handled without causing a nuisance or health hazard. A reduced sludge volume minimizes pumping and storage requirements and lowers overall sludge-handling costs.

Thickening	Digestion	Dewatering		
Sewage sludge	Dewatering	Incineration	Ash	Landfill
	Co-composting	Compost	Land application	
			Sludge disposal options	

Several processes are available for accomplishing these two basic objectives. They include sludge thickening, digestion, dewatering, and co-composting. Typical sludge treatment options are shown in figure.

Incineration is considered as a final disposal option. Co-composting of sludge with garbage and yard waste is discussed in section

Sludge disposal

Widely employed methods for final disposal of waste water sludge have included ocean dumping, land filling, incineration, land application, and sale as fertilizer.

Bio-mining of metals Or bioleaching of ores

Bio-mining or bioleaching is the process of microbial extraction of metals from low grade ores and mining wastes. Applications of biotechnology in bio-mining would resolve an environmental problem, while making valuable metals available to industries. Bio-mining is effectively applied to recover metals from tailing dumps accumulated at the mine site over the years accounting to several million tonnes. *Thiobacillus ferroxidans* is commercially used for the recovery of metals like copper, uranium and gold from low grade ores. Bioleaching can involve numerous ferrous iron and sulfur oxidizing bacteria, including *Acidithiobacillus ferrooxidans* (formerly known as *Thiobacillus ferrooxidans*) and *Acidithiobacillus thiooxidans* (formerly known as *Thiobacillus thiooxidans*). As a general principle, Fe^{3+} ions are used to oxidize the ore. This step is entirely independent of microbes.

The role of the bacteria is the further oxidation of the ore, but also the regeneration of the chemical oxidant Fe^{3+} from Fe^{2+}. For example, bacteria catalyse the breakdown of the mineral pyrite (FeS_2) by oxidising the sulfur and metal (in this case ferrous iron, (Fe^{2+})) using oxygen. This yields soluble products that can be further purified and refined to yield the desired metal. Pyrite leaching (FeS_2):

Pyrite leaching (FeS_2): In the first step, disulfide is spontaneously oxidized to thiosulfate by ferric ion (Fe^{3+}), which in turn is reduced to give ferrous ion (Fe^{2+}):

$$FeS_2 + 6\,Fe^{3+} + 3\,H_2O \longrightarrow 7\,Fe^{2+} + S_2O_3^{2-} + 6\,H^+$$ spontaneous

The ferrous ion is then oxidized by bacteria using oxygen:

$$4\,Fe^{2+} + O_2 + 4\,H^+ \longrightarrow 4\,Fe^{3+} + 2\,H_2O$$ (iron oxidizers)

Thiosulfate is also oxidized by bacteria to give sulfate:

$$S_2O_3^{2-} + 2\,O_2 + H_2O \longrightarrow 2\,SO_4^{2-} + 2\,H^+$$ (sulfur oxidizers)

The ferric ion produced in reaction (2) oxidized more sulfide as in reaction (1), closing the cycle and given the net reaction:

$$2\,FeS_2 + 7\,O_2 + 2\,H_2O \longrightarrow 2\,Fe^{2+} + 4\,SO_4^{2-} + 4\,H^+$$

The net products of the reaction are soluble ferrous sulfate and sulfuric acid.

The microbial oxidation process occurs at the cell membrane of the bacteria. The electrons pass into the cells and are used in biochemical processes to produce energy for the bacteria while reducing oxygen to water. The critical reaction is the oxidation of sulfide by ferric iron. The main role of the bacterial step is the regeneration of this reactant.

The process for copper is very similar, but the efficiency and kinetics depend on the copper mineralogy. The most efficient minerals are supergene minerals such as chalcocite, Cu_2S and covellite, CuS. The main copper mineral chalcopyrite ($CuFeS_2$) is not leached very efficiently, which is why the dominant copper-producing technology remains flotation, followed by smelting and refining. The leaching of $CuFeS_2$ follows the two stages of being dissolved and then further oxidised, with Cu^{2+} ions being left in solution.

Chalcopyrite **leaching**:

$$CuFeS_2 + 4\,Fe^{3+} \longrightarrow Cu^{2+} + 5\,Fe^{2+} + 2\,S_0$$ (spontaneous)

$$4\,Fe^{2+} + O_2 + 4\,H^+ \longrightarrow 4\,Fe^{3+} + 2\,H_2O$$ (iron oxidisers)

$$2\,S^0 + 3\,O_2 + 2\,H_2O \longrightarrow 2\,SO_4^{2-} + 4\,H^+$$ (sulfur oxidisers)

Net reaction:

$$CuFeS_2 + 4\,O_2 \longrightarrow Cu^{2+} + Fe^{2+} + 2\,SO_4^{2-}$$

In general, sulfides are first oxidized to elemental sulfur, whereas disulfides are oxidized to give thiosulfate, and the processes above can be applied to other sulfidic ores. Bioleaching of non-sulfidic ores such as pitchblende also uses ferric iron as an oxidant (e.g., UO_2 + 2 Fe^{3+} ==> UO_2^{2+} + 2 Fe^{2+}). In this case, the sole purpose of

the bacterial step is the regeneration of Fe^{3+}. Sulfidic iron ores can be added to speed up the process and provide a source of iron. Bioleaching of non-sulfidic ores by layering of waste sulfides and elemental sulfur, colonized by *Acidithiobacillus* spp., has been accomplished, which provides a strategy for accelerated leaching of materials that do not contain sulfide minerals.

Several species of fungi can be used for bioleaching. Fungi can be grown on many different substrates, such as electronic scrap, catalytic converters, and fly ash from municipal waste incineration. Experiments have shown that two fungal strains (*Aspergillus niger, Penicillium simplicissimum*) were able to mobilize Cu and Sn by 65%, and Al, Ni, Pb, and Zn by more than 95%. *Aspergillus niger* can produce some organic acids such as citric acid. This form of leaching does not rely on microbial oxidation of metal but rather uses microbial metabolism as source of acids that directly dissolve the metal.

Extractions involve many expensive steps such as roasting, pressure oxidation, and smelting, which require sufficient concentrations of elements in ores and are environmentally unfriendly. Low concentrations are not a problem for bacteria because they simply ignore the waste that surrounds the metals, attaining extraction yields of over 90% in some cases. These microorganisms actually gain energy by breaking down minerals into their constituent elements.

The company simply collects the ions out of the solution after the bacteria have finished. There is a limited amount of ores.

Advantages

Economical: Bioleaching is in general simpler and, therefore, cheaper to operate and maintain than traditional processes, since fewer specialists are needed to operate complex chemical plants.

Environmental: The process is more environmentally friendly than traditional extraction methods.] For the company this can translate into profit, since the necessary limiting of sulfur dioxide emissions during smelting is expensive. Less landscape damage occurs, since the bacteria involved grow naturally, and the mine and surrounding area can be left relatively untouched. As the bacteria breed in the conditions of the mine, they are easily cultivated and recycled.

Ore concentration: Bioleaching can be used to extract metals from ores that are too poor for other technologies. It can be used to partially replace the extensive crushing and grinding that translates to prohibitive cost and energy consumption in a conventional process.

Disadvantages

Economical: The bacterial leaching process is very slow compared to smelting. This brings in less profit as well as introducing a significant delay in cash flow for new plants.

Environmental: Toxic chemicals are sometimes produced in the process. Sulfuric acid and H^+ ions that have been formed can leak into the ground and surface water turning it acidic, causing environmental damage. Heavy ions such as iron, zinc, and arsenic leak during acid mine drainage. When the pH of this solution rises, as a result of dilution by fresh water, these ions precipitate, forming "Yellow Boy" pollution. For these reasons, a setup of bioleaching must be carefully planned, since the process can lead to a biosafety failure. Unlike other methods, once started, bioheap leaching cannot be quickly stopped, because leaching would still continue with rainwater and natural bacteria.

At the current time, it is more economical to smelt copper ore rather than to use bioleaching, since the concentration of copper in its ore is in general quite high. The profit obtained from the speed and yield of smelting justifies its cost. Nonetheless, at the largest copper mine of the world, Escondida in Chile the process seems to be favorable.

However, the concentration of gold in its ore is in general very low. In this case, the lower cost of bacterial leaching outweighs the time it takes to extract the metal. Economically it is also very expensive and many companies once started cannot keep up with the demand and end up in debt.

Oil Degradation by superbug

Although many microorganisms can metabolize petroleum hydrocarbon no single microbe possesses the enzymatic capability to degrade all, or even most of the compounds in a petroleum mixture. Recombinant DNA technology has created a **'superbug'** that is able to degrade many hydrocarbon structures, that is potentially useful in oil pollution abatement programmes. This hydrocarbon-

degrading microbe, *Pseudomonas putida* is the first organism for which a patent has been granted in the U.S.A. Different strains of this bacterium contain a plasmid, which has genes for enzymes that digest a single family of hydrocarbon. These plasmids are designated based on the hydrocarbon they metabolize. Plasmid *CAM* digests camphor, *XYL*- xylene and toluene, *NAH*- naphthalene and *OCT*- octane. By crossing various strains of this bacterium a super bug was created. It carries the plasmids *XYL, NAH* and a hybrid plasmid having *CAM* and *OCT* genes. This multi plasmid bacterium can grow on a diet of crude oil. It has a potential of cleaning up of oil spills as it degrade all the four families of hydrocarbons.

Plastics

Plastics form a major part of global domestic and industrial waste. Not being biodegradable, waste plastic accumulates, adding to pollution. In USA plastic are 7% in weight of all solid waste but 30% of the volume. Standard plastic takes several hundreds of years to disintegrate, over 400 years for the plastic bottles used for mineral water.

Using photodegradable plastic or biodegradable plastic can solve plastic pollution problem. Photodegradable plastic contains an element sensitive to UV rays. Under the effect of solar rays the element is activated and breaks the polymeric chain of the photodegradable plastic. It results in small fragments that are easily digested by microbes.

Biodegradable plastic

Biodegradable plastic is made by adding at least 6% starch and an oxidizing agent (vegetable oil) to the polymers during manufacture. In the biologically active soil environment, the biodegradable plastic is decomposed easily. The metallic salts naturally present in soil interact with the oxidizing agent to form ferro oxides, which attack the polymer bonds and set the biodegradation of plastic in motion. Parallely, soil microbes break up the starch grains (amyloids), which results in an increased attack surface and accelerates the auto oxidation process. The presence of starch reduces the water resistance of plastic. Addition of a fine protective layer to the starch based plastic, make it possible to obtain high degree of water-resistance.