Basic of Fermentation Technology

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Origin and Evolution of Fermentation Process and Fermented Foods

In earliest times, man was plagued with either feast or famine, so any means he could discover to conserve food when it was plenty was a great step forwarded in his survival and his conquest of earth. Since man was of necessity a wanderer and a hunter, he learned about the drying and smoking of meat. Certainly these methods not only conserved his supplies, but they also reduced weight, enabling him to carry more food with him. At the times of discovery of North America by Europeans, most of the Indians were in this stage of development. The discovery of two methods Drying and Smoking just like the invention of wheel perhaps took place by serendipity. Early man might not know why foods spoil he knew they did. Later we can speculate that he discovered the use of salt with drying and smoking.

Man's next discovery for preserving food was the fermentation of foods, although he had no idea what happened when microbial growth occurred, he learned that plant materials and meat could be kept for long periods of time when they have undergone fermentation. It was also essential that he knew how to use salt (a necessary agent that inhibits toxin production from microorganisms in a successful fermentation). Undoubtedly many an early ancestors of man died from botulism or was made ill by Staphylococcus aureus. After the addition of salt in natural fermentation there he knew definite changes in color, odor appearance and taste, which helped the product to be wholesome. Probably the first fermentation was discovered accidentally when salt might have selected certain harmless microorganisms that fermented the product to give nutritious and acceptable food. If we speculate along these lines, we might expect the first fermented food have been fish. With the advent of certain religions in which meat was excluded from diet, the use of salt and fermentation was adapted to certain plant products. For instance Bush (1959) states that Buddhism was well established religion in China and Korea by 4th century. It was introduced to Japan between 500-600 AD. It may very well be the cultivation of Soya beans and their use in food including fermented foods were then introduced in Japan. For centuries Balkan people have enjoyed fermented milk or yogurt and central Asian tribesmen have found equal pleasures in sour camel's milk or Koumiss. The ancient Sanskrit scriptures of India, the Vedas, documented the food value of Dahi- a fermented milk product similar to yogurt. Further evidences for the existence of soured milk as a food in the early times can be found in Bible. The historical, geographical, ecological and dietetic patterns in various regions of the world are reflected in diversity, variety and types of fermented milks in vogue today. These products are generally produced by the intense activity of the Lactic acid bacterial cultures.

Bread that has been known almost as long as agriculture itself, its preparation involves a yeast fermentation. Loaves of bread have been found in Egyptian pyramids built in six thousand years ago. The art of fermented doughs from cereals was practiced before recorded history. This and the production of liquid, fermented mashes from cereals are closely related processes. It is likely that liquid from a fermented mash was drunk as a slightly alcoholic beverage, while semisolid mash was needed into dough and baked. Even today yeast strain used in the production of ale and bread is that from single species of *Saccharomyces cerevisiae.* Until into the middle of the 19th century bakers obtain their yeast from breweries. At that time lager beer strains of *Saccharomyces uvarum (S. carlsbergensis)* were introduced into central Europe and later in the United States. These strains tolerate high osmotic pressure in the dough and bakers were forced to look for another source of yeast. Distiller's yeast that are also strains of *Saccharomyces cerevisiae* perform reasonably well in bread making, but they were difficult to separate from the distilling mash. This led to the establishment of a separate industry that produced baker's yeast on commercial scale for sale to bakers and for home baking. The production of baker's yeast was increased many folds with the advent of Fed-batch culture.

The discovery of fruit fermentation was made so long ago that the ancient Greek believed wine had been invented by one of their gods, Dionysus. The manufacture of wine have been recorded about 3500 B.C. as wine industry of Fertile Crescent that spread west (around the Mediterranean), North (to Hungry, Germany and France) and in the post Columbus period to America, South Africa and Oceania. Romans advanced the art of wine making, but it was an industry of large risks due to spoilage until the mid nineteenth century. The research of Louis Pasteur revolutionized the wine industry. A Mesopotamian clay tablet written in Sumerian-Akkadian about 500 B.C. tells that brewing was an established profession 1500 years earlier. An Assyrian tablet of 2500 B.C. lists beer among commodities that Noah took abroad his ark. Egyptian documents dating back to 4th dynasty about 2500 B.C. describes malting of barley and the fermentation of beer. Kui- a Chinese rice beer has been traced back to 2300 B.C. When Columbus landed America, he found that Indians drank beer made from Corn. According to Weeks (1949) the etymology of word "beer", as we know today indicates it originated from Latin verb 'bibre' (means to drink). Similarly, the Spanish word for beer 'Cervaza' apparently originated from cervisia, which combines Latin word 'Ceres' (goddess of grain) and 'Vis' (vigor). The art of brewing was spread to England by Teutons that settled in Rhine area became Germanic tribe. The major brewing centers were eventually established in Pilsen, Czechoslovakia, Munich, Dortmund, Germany, Burton-O-Trent, England, Dublin and Ireland. American Indians were already making beer from maize but Mayflower Company brought English type beer to America. English Ale beer was used till 1840s, but German Lager beer became more accepted type of beer because of its superior keeping quality.

The process of fermenting sausages was probably one of the earliest forms of meat processing and its manufacturing probably began before written history. The first mention of written history was in 9th century B.C. when it was mentioned in "Homer's Odyssey". The sausage was

called as “Oryae”. The word “Salami” was coined from the product made in Salamis- a Cyprus city destroyed in 449 BC (Pederson, 1979). Sausages eaten by Babylonians, Greeks and Romans were no doubt fermented and dried meat products. Brested (1938) stated that “Caeser’s legions” in Gaul consumed dry sausages. The descriptions of the process of making sausages confirm that Babylonians, Greeks and Romans ate many types of dry sausages. The various regions of Mediterranean developed characteristics sausages e.g. Salamis developed Genoa, Milano and Lambardi types of sausages (Anon, 1938) The Mediterranean countries consumed a highly seasoned non-smoked products classified as Latin type. Non-Europeans countries developed a Roman product, but slightly spiced, heavily smoked, moist and higher in salt content. This product is often referred to as Germanic type. In colder areas sausages are made in the winter months, stored and aged until summer, hence they are called as Summer Sausages. The aging occurs by indigenous flora prompting the growth of Lactic acid bacteria, yeasts and molds in and on the surface of sausages. Early in 20th century bacteria were discovered to be responsible for Lactic acid production and nitrate reduction in sausages. Further research in the microbiology has led to the production of very safe processed meat products and newer products are under the stage of development. Many fermented products have been proved to possess some medicinal values.

Development of Fermentation Process and Industry

Development of fermentation process may be represented by five overlapping stages. Stage I represents the pre-1900 development that is confined to potable alcohol and vinegar. Wooden vats and even fitted with some process control like thermometers (1757) and primitive heat exchangers (1801) replaced the ancient traditional Beer production by Egyptians. In mid 1800s Cagniard-Latour, Schwann and Kutzing demonstrated role of yeast in alcoholic fermentation independently. Pasteur later convinced that pure culture of these microorganisms produces more alcohol than the mixed culture. Methods for isolating and propagating pure yeast cultures were developed in the late 1800s. By the late 1800s and early 1900 generator for the production of vinegar was developed, which was considered as the first aerobic fermentor to be developed. In this method 10% good vinegar was added to the medium as an inoculum that also makes the medium acidic to make it contamination free. Thus in the beginning of 20th century concepts of process control for the fermentation process were developed.

Between the years 1900-1940 the main thrust areas of research were baker’s yeast and organic solvent fermentations. Newer products developed were yeast biomass, glycerol, citric acid, lactic acid and acetone-butanol. Studies indicated that growth of yeast in the fermentation broth leads to oxygen depletion, which results in the ethanol production at the expense of cell formation. Adding more broth in the previous broth can regulate the subsequent growth of yeast cells. This technique now is called as “Fed-batch Culture. Further studies also showed that growth of yeast cells could be improved by sparging air in the fermentation broth. During the First World War Weizmann introduced a concept of Aseptic fermentation the development of Acetone-Butanol fermentation. Steam sterilized hemispherical topped and bottomed vertical steel cylinders were used as fermentors. These fermentors had problems of inoculum development and maintenance of aseptic conditions. In spite of all the hindrances these organic fermentations paved a way for the introduction of aseptic aerobic fermentation technology.

In the Third Stage Penicillin fermentation process was developed, which was a wartime need. This fermentation was very vulnerable to contamination. All the knowledge gained in the previous year’s regarding process control, air sparging, isolation and propagation etc. were applied for the synthesis of Penicillin at a large scale. The development of large-scale extraction process and initiation of strain improvement programme was advancement at that time. Many other fermentation processes were developed at that time like antibiotics, vitamins, gibberellins, amino acids, enzymes and steroid transformations. The fourth Stage (early 1960s) is marked by the production of microbial biomass as a source of feed proteins. Many waste products were considered as a carbon source for the development of microbial biomass. Hydrocarbons as another potential source for carbon for microorganisms were discovered. In this period Jet and Pressure cycle fermentors were developed that eliminated the need of mechanical stirring. Other advantages of these processes were that they can be run continuous and were economic. At this time Batch and Fed batch culture techniques were common in the industry, but their application became short lived because of the development of Continuous Culture. The high standards of the aseptic operation and process controls were achieved by the introduction of computer systems in the fermentation process to minimize the possibility of human error. The fifth Stage in the progress of fermentation process is the introduction of Genetic Engineering and Recombinant DNA technology in the strain improvement programme. These Techniques not only allowed the transfer of genes between unrelated organisms but also enable the extremely precise alteration of genome of a particular organism. The development of further stages in fermentation will depend upon the new advances in this area.

Fermented Foods

Fermented foods form an important part of human diet. Fermented legume and cereal products are especially popular in South East Asia including India, Middle East and Africa. Traditional fermented foods are important elements in the diets of millions of people of particularly in developing countries and the methods for their preparation are simple and inexpensive. Indigenous fermented foods are so prepared that they utilize cheap sources, supply proteins, and enrich starchy diets with vitamins and other nutrients. The exact origin of fermented foods is not known and their

discovery is considered to be purely by chance. The Asians centuries ago knew the art to produce meat like flavors from vegetable proteins. The Indonesians had various methods to introduce meat like texture into the vegetable products. Such foods have a particular place in their diets. Koreans introduced acid fermented vegetables and People of Egypt developed Bread leavened with yeasts while Indians discovered methods for souring and leavening cereal-legume batters. Nearly every nation in the world has one or more fermented milks. Fermented milks are used to restore the natural flora of intestine impaired by disease or antibiotic activity. In many countries cultured milks are widely promoted and credited with health giving properties. Yogurt, Kefir, Acidophilus milk, Bulgarian milk, and Koumiss are a few names that are very popular in many parts of the world and even in western countries. Much interest over the years has been generated in the fermented foods of Asian and African countries because such foods in these countries are prepared traditionally, using simpler technology and equipments. In India preparation of fermented foods has gained a status of small-scale cottage industry that manufactures such foods utilizing natural microflora from staples and surroundings.

Products of Fermentation

A variety of products can be obtained by fermenting different substrates with the help of microorganisms. Fermentation products are the primary or secondary metabolic products of microorganisms that are produced at certain stage of their life cycle. Primary products are Growth Associated products, their concentration in the medium increases, as microorganism grows in the medium i.e. concentration increases gradually in the exponential growth phase of microorganism. Secondary products are Non-Growth Associated products and are produced in the stationary phase of growth of microorganism. The concentration of Non-growth associated products may sometimes become toxic to the microorganism itself e.g. Antibiotics.

Kinetics of Growth Linked Product Formation

The formation of growth Associated product may be described by the equation:

$$dp / dt = qp.x \quad (1)$$

Where p is the concentration of product and qp is the specific rate of product formation. Also the product formation is related to biomass production by the equation as:

$$dp / dx = Yp / x \quad (2)$$

Where Yp/x is the yield of product in terms of substrate consumed.

Multiplying equation (2) on both sides by dx/dt we get:

$$dp / dt = Y_p / x \quad \text{----------(i)}$$

$$dp / dx = Y_p / x \quad \text{---------(ii)}$$

Therefore:

$$dp / dt = Y_p / x \,.\mu \,.\, x \quad \text{----------(iii)}$$

Combining equations (i) and (iii) we have

$$Q_p = Y_p / x.m$$

It may be seen that when product formation is growth associated the specific rate of product formation increases with specific growth rate. When product is Non-growth associated the specific rate of product formation may remain constant over a wide range of growth rates or it may vary in a complex manner. Garden relates the formation of products to substrate utilization or in other way this classification assesses the extent to which the energy producing reactions are coupled to the product forming reactions. This approach is now very much used in studying the continuous process. According to Gaden's Classification product forming reactions fall into following three categories:

Type -I, Type -II and Type III

Type I arise as a result of primary energy metabolism. The desired product results from a carbohydrate substrate e.g. glucose to ethanol, glucose to lactic acid etc. The metabolic roots are serial with μF negative. The kinetic approximation of alcohol fermentation is given by:

$$\frac{d(\text{product})}{dt} = -k_1 \cdot \frac{d(\text{substrate})}{dt} = k_2 \cdot \frac{d(\text{cell mass})}{dt}$$

Negative sign indicates that substrate is decreasing.

In Type II the main product arises from energy metabolism but indirectly e.g. Citric acid fermentation, some amino acid fermentation. The reaction patterns are complex and restricted or abnormal metabolism is involved. The overall free energy change is negative. Such types of products are also called as Intermediate metabolites. Gaden suggests following prototype reaction for the complex dissimilation of

Type II metabolites: (Figure 1)

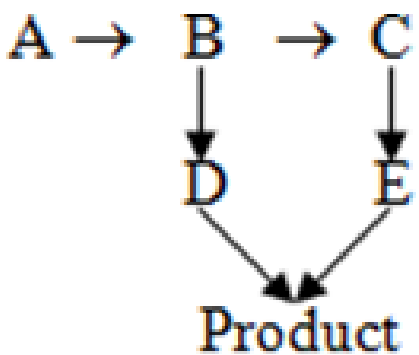


Figure 1

$$\frac{1}{\text{Cell mass}} \times \frac{d\,(\text{cell mass})}{dt} < \text{zero}$$

Type III, Secondary, or Non-growth Associated products

Secondary products result from biosynthetic reactions where the main product does not result from energy metabolism. Cell and metabolic activities reach maximum in the early stages of the life cycle and product formation takes place at the later stages of the life cycle. Oxidative metabolism is low at the time of maximum product formation e.g. Antibiotic fermentation and biosynthesis of Vitamins. In Non-growth associated product formation a period of negative specific growth rate occurs where the terms

This shows that the population has moved beyond the stationary phase for the latter part of the fermentation. Under such conditions dead cell lysis may provide a second nutrient source for the other cells. Fermentation processes are the prime sources of over hundred products for food, chemical and pharmaceutical industries. Various fermentation products are as follows:

Antibiotics: Fermentation industry once dominated by alcohol and solvent making now derives its primary income from antibiotics like *Penicillin, Streptomycin, Vancomycin, Neomycin, Chloramphenicol, and Erythromycin* etc.

Steroids: Transformation of steroids i.e. induction of hydroxyl in 11β *11*β and 16β position, dehydrogenation of 1,2 position and hydrolysis of esters of the 3 hydroxyl group can be achieved by fermentation process. Key steroids in microbial transformation process are Cortisone, Hydrocortisone, and Prednisolone etc.

Enzymes: Many enzymes can be produced by fermentation process. These find application in production of food, chemical and medicines e.g. amylases, pectinases, proteases, cellulases, catalases, invertases, lipases, streptokinases glucose oxidases and collagenases.

Organic Acids: Organic acids find their use in food, chemical and medicines as an acidulant, sequestrate, plasticizer, flavouring and reducing agents. Commonly used organic acids are citric acid, lactic acid, gluconic acid and itaconic acid. Amino acids like lysine is used as food supplement glutamic acid as a flavouring agent and are ascorbic acid as a reducing agent.

Vitamins and Growth factors: Fermentations have been used to produce growth factors for many years. These are used in pharmaceuticals and as food and feed supplement e.g. Riboflavin, vitamin B_{12}. Gibberellin is used in germinating barley and ripening fruits, Xanthophylls produced by algal cultures is added to chicken feed to give color to egg yolks and chicken meat. Torula yeast is added in animal feed as a source of B vitamins is derived by fermentation of waste liquors from paper industry.

Solvents: Many solvents like alcohol, acetone and butanol are prepared by fermentation process. However some of them now are produced by synthetic process.

Polymers: Dextran is the only polymer that is being produced on large scale by fermentation process. It is used as a blood extender and blood thickener.

Miscellaneous: Sorbose (an intermediate in ascorbic acid manufacture), fructose (a liquid sweetener), dihydroxyacetone (a sun-tanning agent) and phenylacetylcarbinol (an intermediate in L-ephedrine synthesis) are a few miscellaneous compounds produced by fermentation process. Spores of Bacillus thuringiensis used as an insecticide for chickens, is also prepared by fermentation process.

Probiotic

A bacterial supplement of a single or a mixed culture of selected non-pathogenic bacterial strains is termed as Probiotic. The term 'Probiotic' was firstly coined by Parker (1974) and originated from two Greek words 'pro' and 'bios' which means 'for life'. Probiotic generally includes bacteria, cyanobacteria, fungi etc. They may be called as normal micro biota or "Effective micro biota". Probiotic, Probiotic, Probiotic bacteria, beneficial bacteria, or friendly bacteria are the synonymously used for probiotic bacteria. According to some recent publications, the mechanisms of action of probiotic bacteria have several aspects: 1) they competitively exclude the pathogenic bacteria or produce substances that inhibit the growth of pathogenic bacteria (e.g. bacitracins and polymyxins produced by *Bacillus spp*.) 2) provide the essential nutrients to enhance the nutrition of the cultured organisms 3) they may directly uptake the or decompose the organic matter or toxic materials in water improving the quality of water in the mediums or in the treatment of water. Beneficial effects of the Probiotic may be mediated by: 1) Neutralization of the toxins 2) suppression of the viable count 3) production of antibacterial substances 4) competition of adhesion sites 5) Alternation of microbial metabolism 6) Stimulation of immunity of the host 7) Accelerate the sediment decomposition by producing organic acids in water treatment 8) production of hydrogen peroxide 9) production of enzymes.

a. Types of Probiotic

i. Non-viable Probiotic –these are dead.

ii. Freeze-dried Probiotic –these will die rapidly upon leaving refrigeration.

iii. Fermentation Probiotic –these are produced through fermentation.

iv. Viable Probiotic–these live with guaranteed shelf life. Guaranteed number of organisms has a protocol for counting and to be very stable and efficacious. Produce many benefits.

b. Bioreactor or fermentor

Bioreactor is a device in which biochemical transformations take place. It is here a less expensive material is converted into a more valuable product or

service is rendered. Even though the term is new but the concept is old. The terms like bioreactors, microbial reactors, fermentors, and biochemical reactors all have the same meaning. Until about four decades ago, fermentation had been practiced as an art with a little engineering input, but with the realization of the potential of this process, the need of its instrumentation and control was felt. With proper instrumentation and control of a bioreactor it is possible to increase the conversion yield and the productivity of a biological product manifolds. The first step in understanding, controlling and optimizing a process is the precise, accurate and timely monitoring of important parameters. The state of the art in automated monitoring is very advanced in mature industrial sectors but even today it is adapting sensors developed for other applications or designing new sensors to satisfy its needs.

c. Measurement system

From system's point of view, a measurement is achieved through the use of a meter or sensor expanding the human senses ability to detect measure and quantify. To control a fermentation process we need to know 1) state of the process within a small time increment i.e. continuous monitoring of the state variables 2) The microorganism's response to any set of measurable environmental conditions i.e. a control model for fermentation. Before describing the various sensors available for a bioreactor, it needs to emphasize the requirements of an ideal sensor. The requirements and characteristics must be met within reasonable limits; it may vary from one case to another.

These are:

i. Reliability-Long term reliability is of great importance and a time of about 2000-3000 hours continuous operation should be attainable for most instruments

ii. Repeatability or Reproducibility -Measurements made under standard conditions should be repeatable from day to day and from laboratory to laboratory

iii. Accuracy- Accuracy is the measure of how close the empirical measurement is to the true value or its conformity to an accepted standard value

iv. Rough and Tough- Sensor should be rugged and repeatedly withstand the conditions of steam sterilization, variety of chemicals besides acidity, basicity, salinity, and water etc.

All the fermentation sensors can be categorized as 1) Physical environmental sensors-to measure the physical process variables and 2) Chemical environmental sensors-to measure the chemical process variables.

d. Methods of Measurement of Process variables

Physical process variables

a. Temperature: It is an important parameter in the biochemical process. This is not true only further reaction itself, but also for auxillary operations such as sterilization and downstream processing. Temperature is usually measured with the help of Resistance thermometers, Thermocouples and liquid expansion thermometers. Risk of contamination is minimal with all these methods. Resistance thermometry prevails because of its accuracy and reliability. Sensors usually are the encased platinum wires. The use of thermocouples is less frequent. Electric measuring signals from both the resistance thermometers and thermocouples can be transferred to control boards. This is not possible with liquid expansion thermometers like mercury or ethanol in glass, which occasionally are employed for direct on the spot measurements. Another method of on the spot temperature indicator is by means of thermo colors that change their colors at certain temperature. They can be applied in the form of thermo foils attached at critical spots. Some details of the devices used to control the temperature are as follows:

b. Mercury in glass thermometers: They may be used in small bench fermentors and are very fragile in nature. That is why their use is restricted. They usually are used enclosed in a pocket which protects the glass. They are used only as an indicator.

c. Bimetallic thermometer: It consists of bimetallic coil surrounded by protective tubing. The coil Winds or unwinds with changes in temperature causing movement of fixed pointer onto it. They are more expansive and less accurate.

d. Pressure bulb thermometer: It is basically a pressure gauge connected by a small bore tubing which may be up to 60m in length to the detecting bulb. The whole system is gas tight and filled with an appropriate gas or liquid under pressure. The movement of the free end of the receiving element can be used to operate a pen on a chart recorder or an electrical or pneumatic control. (Figure 2)

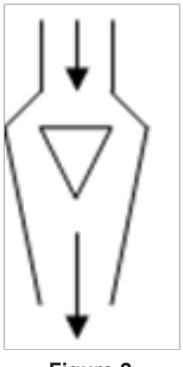


Figure 2

e. Thermocouples: In 1821 Seebak discovered that if a circuit consisting of wires of two dissimilar metals had the junction of the wires maintained at different temperatures, a current flowed through the circuit. This current produced can be measured on a calibrated

instrument or recorder and is a measure of point temperature. At a point therefore by holding the temperature constant at all junctions except one, which is a given circuit, it is possible to measure temperature with reference to the old junction temperature. Thermocouples have limited use because they are normally operated at ambient temperatures.

f. **Electrical resistance thermometers:** Electrical resistances of metals changes with temperature variations. The bulb of electrical thermometers contains a resistance element, a mica framework (for accurate measurement) or a ceramic framework, around which the sensing element is wound. Platinum wires of 100Ω resistance are normally used. The wires are then connected to the measuring element. Reading is normally obtained by a wheat-Stone bridge circuit and is the measure of the average temperature of the sensing element. The electrical resistance thermometers are very accurate, more sensitive to small temperature changes and are very fast.

g. **Thermistors:** These are semiconductors made from specific mixtures of pure oxides of iron, nickel and other metals. Their main characteristic is a large change in resistance as a function of absolute temperature. Temperature reading is obtained with a wheat-Stone bridge. They are relatively cheap and stable.

h. **Pressure:** It is measured by means of conventional pressure gauge. Since the manometer is not in the direct contact with the fermenter contents therefore no sterility problem arises. Often measurement of pressure is not included in the standard equipment, though it may yield valuable information especially with laboratory glass vessels. Here any clogging of exhaust pipe may cause a buildup of a pressure head, and thereby apart from the danger of cracking the glass. Other parameters, such as solubility of gases will be affected. In fermentors containing cultures, which tend to form wall growth, deposits of microbial mass on membranes may lead to errors in the monitoring of the pressure.

i. **Flow Rate:** Gas flow rate is important in aerobic fermentations. Likewise the rate of gas production is of interest for cultures producing biogas. Liquid flow rates must be known for continuous and fed batch processes, where the rate of nutrient feed is an essential variable for efficient operation of the process by means of mass balancing control. Furthermore, knowing the liquid flow rate is necessary to control the addition of corrective liquid feed streams, such as amount of base or acid consumed for pH control or the amount of antifoam input. Flow rates are mainly measured by the following devices: 1) Floating body flow meter 2) Differential pressure flow meter 3) Rotating flow meter 4) Electromagnetic flow meter Floating Body Flow Meter Impeller Speed For stirred tank fermenter, impeller speed is an important operating variable, which is very often kept constant. It is usually measured by monitoring the number of revolutions per unit of time. Outside the aseptic area, the impeller speed is measured with the help of a device called Tachometer. (Figure 2)

j. **Power Input:** The power consumption of agitators depend on stirrer speed and physical properties of the stirred fluid especially on its viscosity, which may change drastically during batch fermentations e.g. in some processes for the production of antibiotics like penicillin viscosity changes take place. In large-scale fermentors, the consumption of electric energy as determined by a Wattmeter, yields useful information on the input of agitating power when friction losses in the stuffing box, seals, and motor are accounted for. A direct measurement of agitation power is possible by using Torsion dynamometer or Strain gauges. The latter method is an accurate method.

k. **Foam:** It is a nuisance occurring in most fermentation broths. It may be caused by surface-active metabolites (proteins, polysaccharides etc.), components of the medium, or by cells. Two types of foams have been distinguished 1) Soft foam 2) Hard foam Soft foam is unstable while hard foam is stable. Foam of either form must be suppressed in order to prevent the contamination, clogging of the exhaust system including its measuring devices and loss of culture broth. Foam destruction can be achieved by mechanical or by chemical methods (antifoaming agents). Often both the methods are used in combination. Foam control necessitates its detection. This can be achieved by employing sensors mounted inside the fermenter above the liquid level. Examples of the various probes used are: Electric conducting probe, Capacitance probe and Heat conducting probe.

l. **Volume:** Information on liquid volume is essential in liquid flow control in the filling of the vessel with medium or in continuous culture feed in the continuous culture, which ultimately affects the metabolic activity of the inoculum culture? There are two systems generally used for the on-line volume determinations: 1) liquid level sensors and 2) weight or mass measurement devices. Capacitance probes measures the change in capacitance when liquid level changes in small-scale fermentors. In large-scale fermentors ΔP measurement method is generally used, where a flush mounted diaphragm pressure cell is located in the base of the vessel for liquid height and hence the volume measurement.

m. **Weight/Mass:** Scales of various types determine weight or mass of the liquid. In this method the vessel is suspended on a scale and the combined weight of the vessel and strain type gauges electronically measure liquid. This method cannot be applied to the fixed existing installations.

Chemical process variables

a. **Exhaust gas analysis:** The concentration of carbon dioxide in the exhaust gas from cell reaction is indicative of the respiratory activity and fermentative activity of the inoculum culture and hence is one of the most useful and widely applied measurement methods in the monitoring and controlling a cell bioreactor. Using an Infrared spectrophotometer, gas chromatography and mass spectrometer most commonly controls carbon dioxide content in a bioreactor. Gas stream oxygen partial pressure is usually measured using a paramagnetic analyzer. Care should be taken in both the cases that water vapors should be eliminated from the exhaust before feeding them into the analyzer. The paramagnetic analyzers are quite sensitive to small changes in total atmospheric pressure so they require simultaneous monitoring of barometric pressure for compensation in oxygen analysis. Apart from water vapors the samples should also be free from dust particles, aerosols and oil droplets.

b. **Dissolved gases and volatiles:** Dissolved oxygen and carbon dioxide are both important variables in fermentations. These are normally determined by 1) Electrochemical methods 2) Fluorescence quenching and 3) Mass Spectrometry Electrochemical Methods- Electrochemical determination of oxygen and carbon dioxide in fermentation media is performed by means of special sterilizable electrode. Analysis by this electrode is based on detecting the amount of oxygen diffusing from the liquid membrane into an Amperometric or Polarographic measuring cell. Amperometric principles are most frequently used. In Polarographic type oxygen electrode a constant voltage is applied between cathode and anode. At cathode the oxygen that has diffused into the cell is reduced to hydroxyl ion as shown below:

Cathode: $- O_2 + 2H_2O + 4\ \bar{e} \rightarrow 4\ OH^-$

Anode: $4\ Ag + 4\ Cl^- \rightarrow 4\ AgCl + 4\ \bar{e}$

The response of the probe is proportional to the oxygen activity in liquid. Since at equilibrium i.e. for a saturated liquid the activity of a solute is directly related to its partial pressure (fugacity), the readings of electrochemical process are commonly given as percent partial pressure or saturation.

In Potentiometer probe same principle of oxygen diffusivity is used and this diffused oxygen is reduced at cathode surface according to the same above equation:

Pt

$\frac{1}{2}\ O_2 + H_2O + 2\ \bar{e} \rightarrow 2OH^-$

The reaction at anode in galvanic electrode is as follows:

$$Pb \rightarrow Pb^{2+} + 2\ \bar{e}$$

This reaction competes with the cell from which a small amount of current is drawn to provide a voltage measurement, which in turn is correlated to oxygen flux reaching the cathode surface. Fluorescence Quenching- For medical investigations so called Optodes has been developed for oxygen and carbon dioxide determinations. In this, the sensitive element is the membrane into which a fluorescence indicator (Pyrene butyric acid or β- methyl belliferon purine) has been incorporated. This membrane is brought in contact with the broth. The fluorescence quenching in indicator is indicative of the presence of oxygen or carbon dioxide. This method does not consume oxygen or carbon dioxide.

c. **pH:** pH value is an important indicator of the state of the state of biochemical process. The automatic addition of alkali or acid to fermentation broth can be achieved by techniques already in use in other chemical industries but special electrodes have been developed for use in fermentation industry. The half-cell of the glass electrode was composed of Ag/AgCl saturated with KCl. The solid KCl increases the mechanical resistance of the glass particles of KCl on the glass surface during heat sterilization and cooling of the electrode. Other half of the electrode is composed of the same material as the glass electrode, asbestos or porcelain cylinder being used as the junction material. To ensure good insulation, both the glass and reference electrode were mounted in Teflon gaskets and silicone rubber washer were fixed. The internal resistance of the electrode is 300-500meg Ohm. Steel sleeves provided with several holes to allow free passage of broth protect both of these electrodes.

d. **Redox potential:** Another method obtained with electrochemical method is the Redox potential measurement method. Every redox system consists of two components, one is oxidized by electron donation and the other is reduced by electron acceptance. In such a system, an electrochemical potential can be measured by means of an unprotected electrode consisting of a noble metal (Au/Pt), the composition of which is chosen on the basis of relation of donor to the acceptor. In a fermentation system/culture, a great number of redox systems are present simultaneously. Accordingly an exact interpretation of signals from the redox potential measurements cannot be given. It is for that reason that some scientists suggested to rather name this potential as Platinum-electrode potential instead of redox. The competing donors i.e. oxygen and glucose that are present in a fermentation system may serve as a typical example. In spite of the difficulty of interpreting the results, measurements of redox potentials permit an important insight into the course of fermentations. Sterilizable Platinum electrodes are commercially available. They either contains built in reference electrode (Ag/AgCl) similar to pH electrode or they are used in combination with pH measurement making use of the same reference electrode. The amplifier for redox measurements is of the same type as in conventional pH meters. As in the latter case, the built in electrode is sterilized together with the fermenter. Since no membrane is required so any

special sterilization problem exists in these electrodes. The signal of the redox meter is influenced by pH. This can be tolerated because fermentations are usually run at constant pH.

e. **Enzymatic analysis of substrate:** Enzymatic analysis allows very specific determination of many organic compounds. This group of methods takes advantage of the ability of enzymes to react selectively with well-defined compounds or rather chemical structures; organic compounds present in fermentation media (substrates and metabolites) can be analyzed. Usually this is performed offline with samples taken from fermentors but methods for online enzymatic analysis have also been developed. The procedure consists of determining the conversion of the enzyme-catalyzed reaction with the respective substrate for analyzing one of the products either calorimetrically or electrochemically. In applying online enzymatic analyses to sterile fermentations, special difficulties arise because conventional sterilization techniques apply steam at 120˚C that will destroy the enzymes. One solution to this is to employ dialysis through which a recycled sample stream of broth is conducted. Components diffusing through the dialysis membrane can then be monitored continuously by means of enzymatic analysis. Such systems have been developed for measuring glucose, saccharine and lactose.

f. **Ion specific electrodes:** Not only carbon source and other organic compounds but also inorganic salts (N, P, S, K, Mg, Ca, Na, and Fe) are essential constituents of fermentation broths. Ion specific electrodes have been proposed for a number of these ions. Some electrodes of this type are commercially available for offline measurements. There is little known about the online measurement using ion selective electrodes. Ion selective electrodes are Infact potentiometer electrodes applying different principles e.g. for measurement of Na+ glass electrodes with glass membrane especially sensitive to Na+ are employed. In some electrodes organic membranes are employed while in others enzymes may be incorporated. For these types of electrodes sterilization may be the problem.

How Temperature and pH Affect the Growth of Microorganisms

Effect of temperature on the growth of microorganisms

Bioprocesses of microorganisms are heavily affected by the temperature. The cell temperature must become equal to the culture temperature. Temperature affects the rate of cell reactions, the nature of metabolism, the nutritional requirements and biomass composition. The temperature coefficient of growth rate is denoted by Q10 value (It is defined as increase in growth rate per 10°C rise in temperature e.g. If Q10 is equal to 2 that means there is two fold increase in growth rate per 10 °C rise in temperature. Growth rate approaches zero at 10 to 25 °C below the optimum temperature. Chemical reaction rates are related to temperature by Arrhenius equation:

$$K = A\, e^{-E/RT}$$

Where K = reaction rate; A= Arrhenius constant; R = Gas constant; T = Absolute temperature; E = Activation energy or temperature characteristics.

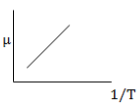
Taking log on both sides we have:

$$Log\ \mu$$

$$1 / T$$

Plot of Log K against 1/T should be a straight line with slope of E/2.3 RT. If we substitute specific growth rate 'μ' for reaction rate K in the above equation then converting it to straight line equation and plotting log μ against 1/T, keeping the value of E constant we find a straight line with slope of E/2.3RT. The Q10 value also varies inversely as the temperature varies from normal as

$$Q_{10} = E \,.\, 10 / 2.3RT\ (T+ 10)$$



The activation energy is a valuable constant as it can be used to predict the effect of temperature on growth rate over the normal temperature range. Changing in the activation energy indicates that differences in rate controlling reactions or in the metabolic regulations can occur. Temperature range for growth of individual bacteria extends over about 35°C. Extreme psychrophiles grow between -5 to 30°C and extreme thermophiles 55 to 90°C. Decrease in growth rate at high temperature is due to disruption of metabolic regulations or death of cells by protein denaturation. When death of cells occur the growth rate of the viable biomass (X) is given by:

dX/dt = (μ -K) X where μ = specific growth rate; K = specific death rate and X = biomass

Death rate becomes dominant if at high temperature activation energy for death exceeds that for growth. Increase in temperature causes breakdown of protein structure so the affinity for substrate and enzyme regulators will be affected. Thermophiles possess proteins with exceptional heat resistance. Temperature also affects the nutrient requirements, lowering of the growth temperature causes small increase in the growth yield from carbon and energy source. The pathways of metabolism of carbon and energy source can be temperature sensitive e.g. *Lactobacillus brevis* ferments glucose by heterolactic pathway at 24°C but at 32oC requires fructose as hydrogen acceptor for glucose fermentation. Growth factor requirements also change with temperature e.g. *Yersinia pestis* requires different amino acids and vitamins at growth from 37 °C to that at

28°C. Temperature affects the product formation e.g. over production of riboflavin by Ashbya gossypii requires growth of the microorganism at 28°C than at its normal temperature because growth at low temperature causes breakdown of normal regulation of synthesis of enzyme system which produces the riboflavin. Similarly the optimum temperature of production of Penicillin is lower than that of normal growth temperature. Temperature affects the microbial composition as RNA content of bacteria or yeasts increase several folds on decreasing the temperature. Yeast lipids increase their unsaturated fatty acids when temperature is lowered. Antigenic composition of bacteria varies both qualitatively as well as quantitatively with temperature e.g. virulent Yersinia pestis is produced at 37°C but not at 25°C.

Mechanism of temperature effect

The effect of temperature can be explained as: 1) Dependence of structure of cell components on temperature, 2) Activation energy required for the reactions to occur inside the cell which in other term affects the regulatory mechanisms of the cell, cell composition and permeability functions.

Effect of pH on the growth of microorganisms

The influence of [H+] on biological activities is related to either hydrogen ion concentration or hydrogen ion activity (ah). These two parameters are proportional as:

ah = f [H+] where f is the activity coefficient which may vary with the ionic strength and other factors. The glass electrodes respond to hydrogen ion activity so that strictly pH = -log (ah) and [H+] can be substituted for hydrogen ion activity. It can only be possible when activity coefficient is one. In dilute media solutions f approximately is one. But this may be far from true when media are strong salt solutions. As far as cell properties are concerned hydrogen ion activity is more meaningful parameter so that it is appropriate to express the effect of hydrogen ion concentration in terms of pH. Plasma membrane is not freely permeable to hydrogen ions or OH- ions. So that intracellular and extracellular hydrogen ion concentrations do not necessarily equilibrate and a gradient of hydrogen ion across the plasma membrane is established. According to chemiosmotic theory this gradient of hydrogen ions together with membrane electric potential makes a proton motive force that derives the membrane reactions.

Lactic Acid

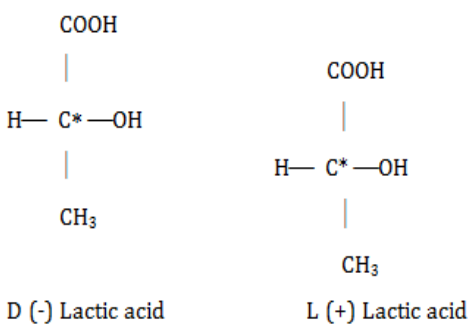
Scheele (1789) first isolated lactic acid from sour milk. The studies on the physical and chemical properties have shown that the compound occurs in two isomeric forms and as a mixture of the two isomeric forms. Lactic acid is also produced in muscles. Many microorganisms produce lactic acid by the fermentation of sugars. The structural formula of Lactic acid is CH_3 -CH (OH)–COOH.

Technological development

Lactic acid was the first organic acid to be manufactured industrially by the fermentation process. The first commercial production of lactic acid in USA by microbiological process took place in 1881 as its calcium salt Calcium lactate. The plant for its manufacture was built in Littleton, Massachusetts, but little is known about its process. The Clinton processing Company, Clinton Iowa is the only manufacturer using fermentation process for the production of lactic acid in United States.

i. **Isomers:** Lactic acid of commerce is the L (+) isomer, D (-) isomer or any possible mixture of the two. The entire range of isomers has been found. The mixture of the two forms of isomers is called as DL mixture or racemic mixture. DL mixture is optically inactive form. Microorganisms differ in their ability to produce either D (-) lactic acid or L (+) lactic acid or racemic mixture and the particular acid formed are the characteristic of individual microorganism. From industrial standpoint the lactic acid recovered from fermentation broth usually is the racemic mixture because fermenting microorganisms or contaminants like Lactobacillus plantarum produce an enzyme known as Racemase that converts either of the optically active isomer to optically inactive racemic mixture. Some trace impurities in the medium also have been reported to bring about racemization. The Racemase are known to be lactic dehydrogenises that maintain equilibrium between lactic acid isomers and pyruvic acid. When both dehydrogenase enzymes are present, racemization occurs.

ii. **Microorganisms:** Many types of microorganisms have been isolated that accumulate lactic acid or lactates in the culture solutions e.g. Lactic acid bacteria, algae, molds, yeasts and phycomycetous fungi. Apart from many Lactic acid bacteria, mold Rhizopus oryzae has been found to produce lactic acid comparable to homofermentative lactic acid bacteria from glucose. Lactic acid bacteria fall under two main groups: (1) Homofermentative Lactic acid Bacteria (2) Heterofermentative Lactic acid Bacteria



iii. **Homofermentative lactic acid bacteria:** These bacteria produce lactic acid as the major or sole product of glucose fermentation. The homofermentative

pattern is observed when glucose is metabolized but not necessarily when pentoses are metabolized, for some homolactics produces acetic acid and lactic acid when utilizing pentoses. Also, the homofermentative character of homolactics may be shifted for some strains by altering cultural conditions such as glucose concentration, pH and nutrient limitation. The homolactics are able to extract about two times as much energy from a given quantity of glucose as are the heterolactics. They are less important in producing flavor and aroma components e.g. acetaldehyde and diacetyls in food products. They possess the enzymes aldoses and hexose isomerase but lack phosphoketolase. They use EMP pathway to produce two molecules of lactate per glucose molecule. Examples of homofermentative lactic acid bacteria are: All members of genera *Streptococcus, Pediococcus* and some genera of *Lactobacilli* like *L. delbrueckii* Homofermentative lactic acid bacteria are very important for the production of Lactic acid industrially.

iv. **Heterofermentative lactic acid bacteria:** They produce some lactic acid, but at the same time, they also produce carbon dioxide, ethanol, and acetic acid and trace amounts of a few other products. These organisms are of little use for industrial lactic acid fermentations because too much of the substrate carbon is directed towards products other than lactic acid. The end product differences between homo and Heterofermentative lactics when glucose is attacked are a result of basic genetic and physiological differences. The heterolactics have phosphoketolase pathway but do not possess aldolase and hexose isomerase. Instead of EMP pathway such organisms use Hexose monophosphate shunt as energy pathways. Heterofermentative bacteria are very important for the production of flavor and aroma components like diacetyls in food products. Examples of Heterolactics are: All species of genus Leuconostoc, and some species of Lactobacilli.

Microorganisms for Commercial Production of Lactic acid

The microorganisms used for commercial production of lactic acid depends upon the raw material to be fermented, but the most common bacterium used for this purpose is *Lactobacillus delbrueckii*; it is employed in fermentations utilizing corn dextrose medium. Although increasing use is being made of a flat sour *Bacillus coagulans* yet *Lactobacillus bulgaricus* is used for the production of lactic acid from whey because it utilizes lactose as a carbon source. *L. pentosus (L.plantarum)* is recommended for use in spent sulfite liquor, as it is able to utilize pentoses. L. brevis is used when medium contains hydrolyzed corncobs, cotton seed hulls etc. Other homofermentative species of potential industrial importance are *L.casei, L.leichmannii*, and *Streptococcus lactis*. These are facultative anaerobes and can withstand some oxygen. *Streptococcus lactis* is particularly useful under such conditions.

Medium

The fermentation solutions usually contain hydrolyzed starches or dextrose syrup, although D-glucose, maltose, lactose or sucrose can also be fermented. It is advantageous to start with a relatively simple medium or mash in order to facilitate recovery of the product. Crude carbon sources are generally avoided because the impurities interfere with the recovery and purification procedure. The sugar concentration in the medium should not be more than 12-15% because Calcium lactate produced at high sugar concentration tends to crystallize from the medium late in the fermentation, thereby slowing the fermentation process. Nitrogen sources are added in small amounts and are usually inorganic in nature e.g. Ammonium phosphate. This is because impurities in the nitrogen sources might interfere in the recovery and purification procedure. Calcium carbonate (10%) is added to neutralize the lactic acid produced because lactic acid bacteria cannot tolerate high acid concentration. Lactic acid bacteria have complex requirements of B-vitamins. This ordinarily is met by enrichment of the culture medium with crude vegetable materials. Malt sprouts are commonly used vegetable materials, but if they have been overheated in drying, they lose some of their value as a nutrient for *Lactobacilli*.

Production of Lactic Acid

Today much of the lactic acid is produced by the hydrolysis of lactonitrile, a byproduct of another process. Only a few companies in the world are producing lactic acid by fermentative process.

Equipment

Lactic acid is very corrosive to metals therefore wooden fermentors are used in most of the plants. These fermentors may be uncovered or covered with loosely fitting wooden lids. They are to be steamed empty before charging. Fermentation solutions are pasteurized or sterilized by passing it through a steam jacketed heat exchanger. Contamination of culture solutions sometimes by *thermophilic Clostridia* results in the production of some butanol and butyric acid. Such contaminated lactic acid could only be sold to leather tanners for delining of hides. A very pure lactic acid is required for manufacturing of plastic. Such grade of lactic acid is called Plastic grade lactic acid.

Inoculum

Cultures of *L. delbrueckii* are transferred from test tubes through successively larger culture vessels, held at 45-55 °C. Each stage of the culture build up requires 16-18hours and slight excess of Calcium carbonate is required at each stage. Inoculum volume should be 5% of the volume of fermentation solution.

pH

An excess of Calcium carbonate keeps the pH in the range of 5.5-6.5. The pH necessary varies with the composition of culture solution but it is controlled by continuous

neutralization with the slurry of Calcium hydroxide between 6.3-6.5. Fermentations utilizing grain may resist increase in pH with the buffering capacity of mash.

Aeration and agitation

The medium is not aerated but agitation is done to keep the Calcium carbonate in suspension. Fermentation Time and Fermentation Temperature: The fermentation temperature is adjusted to 45-50 °C and varies the type of organism used. Same is the case with fermentation time. It is usually completed in six days or less depending on the time required by the organism to deplete the sugar in the medium. Fermentation time is usually 5-10 days. It is important that residual sugars be reduced to 0.1% or less during the fermentation because residual sugars make recovery of better quality lactic acid difficult.

Yield

Commercial yields are 93-95% by weight of glucose supplied. Recovery yields vary with the various recovery processes and product grades.

Product Recovery and Grades

Technical grade lactic acid

Calcium from the fermentation solution is precipitated as Calcium Sulfate, filtered, and filtrate is evaporated to 35-40% lactic acid. Now more Calcium sulfate is precipitated, filtered, and filtrate is evaporated to 44-55% total acidity. It is then crystallized.

Food grade lactic acid

It is a pale yellow, straw colored solution of about 50% total acidity. Calcium in this case is precipitated as Calcium sulfate, precipitates are washed, washed water is combined with the filtrate, filtrate is bleached with activated carbon and it is then subjected to evaporation firstly to 25% solids, again bleached and then secondly evaporated to 50% solids, bleached finally to produce an off colored product. Impurities like Iron and Copper metals are removed by adding Potassium ferrocyanide in the filtrate of first filtration.

Plastic grade lactic acid

It is a colorless product. Product is recovered by etherification with methanol after concentration or by solvent extraction with isopropyl ether followed by re-extraction of isopropyl ether with water.

Pharmaceutical grade lactic acid (Lactic Acid USP)

It is a colorless product with 85% total acidity and 76-78% concentration. It contains 2-3.5% volatile acids 0.5-1.0% ash content.

Uses of Lactic Acid

In the foods, lactic acid is added to acidify jams, jellies, confectionary, sherbets, soft drinks, extracts and other products. It is added to brines for pickles and olives and to fish to aid preservation. Its addition makes milk more digestible to infants. Calcium lactate is an ingredient of some baking powders. In tannery, it is used for washing of hides. In textile industry it is employed for fibre washing. Lactic acid is used in the preparation of medicines. It is also used as a laboratory reagent and a research tool.

Citric Acid

Scheele first isolated Citric acid in 1784 from Lemon juice by crystallization process. Members of citrus family of fruits especially are rich in this organic acid, but citric acid is also found as a natural constituent of a variety of fruits. Citric acid extracted from fruits is designated as Natural Citric acid in contrast to the Citric acid produced by Microbial Fermentation process. Citric acid can also be prepared synthetically but no equivalent synthetic process has been invented to the microbial fermentation.

Microorganisms

Many microorganisms like molds (*Aspergillus niger, A. awamori, Penicillium janthinallum, Trichoderma viridae, Mucor piriformis*, etc.), yeasts (*Candida lipolytica, C.tropicalis, C.citrica, Hansenula, Rodotorula, Pichia, Torulopsis* etc.) and bacteria (*Bacillus licheniformis, Bacillus subtilis, Brevibacterium flavus, Corynebacterium species*) have the capability to produce citric acid. Commercially spores of Aspergillus niger are employed to produce citric acid.

Methods of fermentation

Fermentation of Citric acid is carried out by any of the following methods 1) Stationary or Surface culture, 2) Submerged Culture, 3) Solid State Culture, 4) Continuous Culture, 5) Multistage Culture process, 6) Semi-Continuous Culture process.

In stationary or surface culture, sterile nutrient medium with sugar is added into stainless steel or high-grade aluminum trays sterilized with formaldehyde or sulfur dioxide. Spores of *Aspergillus niger* are inoculated and incubated at 28-30 °C for 8-12 days. Submerged process consists of two phases i.e. growth phase and productive phase. In Growth phase medium is inoculated with spores *of A. niger*, after 3-4 days mycelium is separated from solution and added to the fermentation medium and fermentation is allowed to occur for 3-4 days. During the period production of the Citric acid takes place. This phase now is called as Production phase. In Solid state Culture as described by Calm (1935), fermentation medium is impregnated in porous solid materials like sugarcane baggase, potato or beet pulp, or pineapple pulp in an appropriate ratio, sterilized and then inoculated with a suspension of fungal spores and incubated at 25-30 °C for 6-7 days. In Continuous or multistage culture, the medium is replaced after 24 hours and that medium goes to second fermenter. It is then

aerated by gradually increasing the amount of air. In Semi-Continuous Culture, the whole medium is not replaced but a part is replaced.

Medium

Components of the medium varies with the type of the process used because it has been seen that the strains that give good result in surface culture do not perform better in submerged culture. Fungi have been seen to give much better results in simple synthetic media. A variety of carbon sources are being used these days, which include Sucrose, Citrus molasses, Cane juice, Starch from various sources, Cane or Beet molasses. The initial sugar content determines the amount of citric acid produced by *A. Niger*. Normally 15-18% sugars are added into the medium. Concentration of sugar more than 15-18% leads to greater amounts of residual sugars that make the process uneconomical. When the sugar concentration is lower than the above percentage, it leads to lesser yield of citric acid as well as accumulation of oxalic acid. Molasses contains 50-60% sucrose and is of three types: 1) Black Strap molasses- it is obtained from the last stages of crystallization of sugar. 2) Refinery molasses- it is obtained at the second stage of refining sugar. It contains 48-50% sucrose and has high ash content. 3) Invert or High-test molasses- it is partly inverted cane juice syrup from which no sugar has been extracted. Molasses is diluted to sugar concentration of 15-20% and pH adjusted to 5.5-6.5 with sulfuric acid. Molasses is added with other required nutrients and mixture is sterilized for 30 minutes. For the growth stage of *Aspergillus niger*, the organism needs major elements (C, N, P, S) and trace elements. The type of inorganic source and its concentration affects the performance of the fungus. Inorganic sources like Ammonium sulfate prolong vegetative growth. Ammonium nitrate shortens the growth phase. Ammonium nitrate in concentration greater than 0.25% accumulates more of oxalic acid whereas sodium nitrate at concentration 0.4% delays the onset of production phase and vegetative growth also increases. Phosphate also affects the citric acid yield. High phosphate concentration promotes growth and there is less acid production. Therefore phosphate concentration in the medium should be kept at 0.1%-0. 2 %. Trace elements (Fe2+, Cu2+, Zn2+, Mn2+ and Mg2+) are necessary for *A. niger*. Mg2+ is necessary for a variety of enzyme reactions in the cell. It is required for growth and production phase. Its optimum concentration in the medium should be 0.02-0.025%. Fe2+ and Zn2+ have critical role to play in the growth and production of citric acid. Both are essential for the production of citric acid in low concentration because their high concentration allows vegetative growth. Iron also has deleterious effects on the fungus but its action is counteracted by copper ions. Zn2+ at concentration 1-2 µM allows growth phase but its less concentration restricts growth. Its excess in the citric acid producing culture reverses the production phase. It also has its indirect role in the functioning of cAMP that enhances the production phase. To remove the excess of trace elements from the culture medium various methods adopted are: 1) Pretreatment of the raw materials. 2) Ion exchange resins. 3) Development of resistant varieties of fungus. The raw materials are treated with Potassium ferrocyanide to reduce the concentration of iron ions in molasses. The chemical is either added directly in the medium or the molasses is treated with its high concentration. Raw materials are also sometimes treated with chelating agents like EDTA, activated charcoal or polythene amine. Chemicals of quaternary ammonium compounds category like Diiosbutyl phenoxy ethyl dimethyl benzyl ammonium chloride and Triton-B are also used.

pH

Fungus can tolerate high concentration of acid therefore calcium carbonate is not added in the medium. The initial required pH of the medium depends upon the carbon source used as follows: Glucose or clarified molasses pH = 3.0, Crude molasses pH = 5-6, Decationized molasses pH = 1.4-2.8, Sucrose pH = 2.0-3.0. pH is adjusted with HCl, H_2SO_4 or NaOH.

Additive and stimulants

Methanol is usually used to increase the yield at a concentration of 3-4%. At this level it retards growth, delays sporulation and increases citric acid yield. This is added before inoculation. It has some role in the conditioning the mycelium without impairing their metabolism. It is thought that it increases the tolerance of the fungus towards the harmful effects of the trace elements. Other additives like hydrogen peroxide, Methylene blue, Naphthaquinone, aromatic amides, esters of dichloroacetic acid, sodium sulfite, crysyllic acid, glycerol, vegetable oils (corn oil, almond oil and peanut oil) and cAMP are added as stimulant to increase the yield.

Antifoaming agents

Antifoaming agents like Octadecanol (0.75% solution) or Antifoam AE (silicone oil) are added in the medium. To control contamination by bacteria Pentachlorophenol ate, formic acid, tetracycline's, and 3-furaldehyde semicarbazone are added in the medium.

Biochemistry of Citric Acid Formation

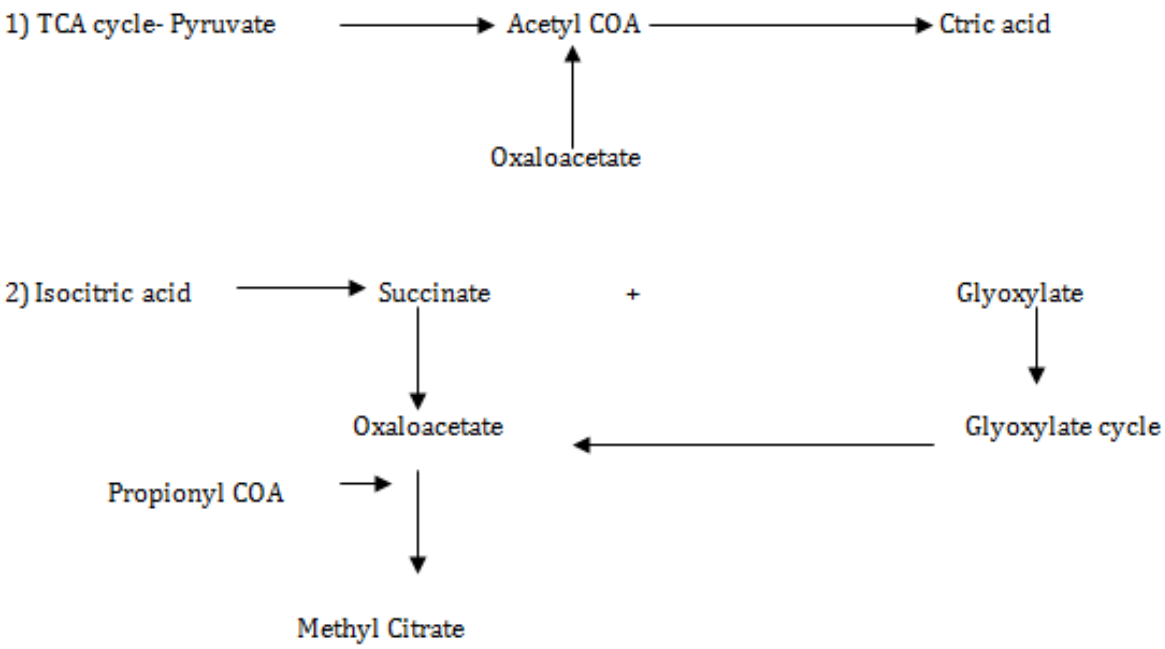
Citric acid is formed via following pathways: (Flow Chart 1)

Citric acid accumulates in culture solutions of pH 1.8-2.0. In fungi different metabolic pathways are involved in the production of citric acid but 78% of the citric acid is produced by the involvement of glycolysis and Tricarboxylic acid cycle. The acetyl COA derived by EMP pathway condenses with Oxaloacetate of Kreb's cycle to produce citric acid. Since it has been observed that, during accumulation of citric acid, *A.niger* demonstrates decreased activity of condensing enzyme and almost no activity of Isocitrate dehydrogenase and aconitase therefore another theory of its production came into light. In this pathway, glucose first splits up into

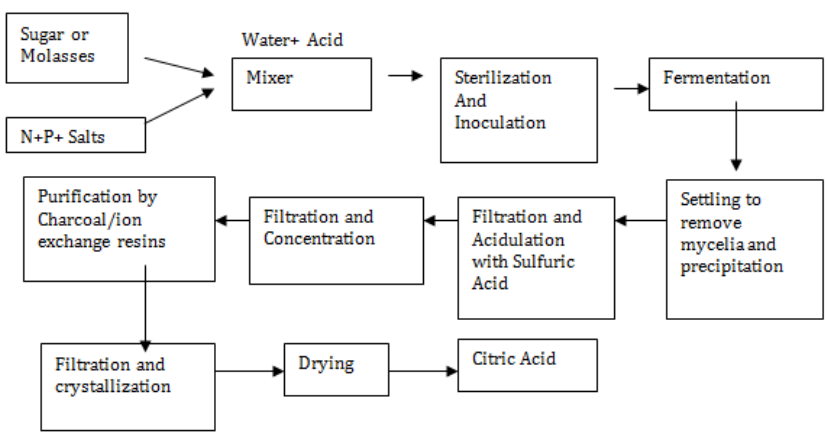
two 3-Carbon fragments followed by decarboxylation of other fragment to yield a 4-Carbon compound. The 2-C and 4-C compounds then combine to yield citric acid. In methyl citrate pathway Propionyl COA formed through the β-oxidation of n-alkanes condenses with Oxaloacetate to yield methyl citrate.

Product recovery

The crude fermented liquor is subjected to filtration to remove mycelia of fungi. Calcium citrate is precipitated from the clear liquid by adding slurry of calcium hydroxide (add hydrated lime 1 part for every 2 parts of liquor added over one hour period and temperature raised to 95°C) and heating the mixture to 80-90°C. Calcium citrate is filtered off on conventional filter and the filter cake is transferred to a tank called acidulator where it is treated with Sulfuric acid to precipitate calcium sulfate, which is then filtered. The dilute filtrate containing citric acid is purified by treatment with activated charcoal and demineralized by passing it through ion exchange resins. The purified solution is evaporated in a circulated granulator or circulating evaporator called crystallizer. Crystals are removed by centrifugation. Citric acid so prepared is subjected to recrystallization. The remaining mother liquor is added into the main stream prior to limming and decolorization (Flow Chart 2).



Flow Chart 1



Flow Chart 2

Single Cell Protein (SCP)

Definition -The dried cells of various groups of microorganisms like Bacteria, Yeasts, Molds, Higher fungi and algae that have been considered for food or feed are collectively referred to as Single Cell Protein. C.L. Wilson in Massachusetts institute of technology coined the term SCP in 1966. People have eaten certain microorganisms as a portion of their diet e.g. Top fermenting yeasts (*Saccharomyces spp*.) was recovered as a leavening agent for bread as early as 2500 B.C. Fermented milks and Cheeses produced by Lactic acid bacteria of genera *Lactobacilli and Streptococci* were consumed by early Egyptians and Greeks and reached a higher state of development during the Roman era (100-50 B.C.). Pharaohs of Egypt prized wild mushrooms as a delicacy. Romans regulated the grading and selling of mushrooms. People of Chad regions of Africa and Aztec in Mexico have eaten *Spirulina spp*. (Blue green Algae) for many generations. The purposeful cultivation of microorganisms for direct use in human food or animal feed is a fairly recent development. Considerable efforts have been expanded since World War-II to develop mass cultivation of microbial cells.

Nutritive value and use of SCP

Nutritive value of SCP varies with the microorganism used. Protein digestibility of SCP is expressed as percentage that ranges from 65-96 for various cultures. Protein efficiency ratio ranges from 0.6-2.6. Food yeasts are high in proteins and vitamins of B-12 category but may be deficient in some amino acids such as cysteine and methionine. The UN protein advisory group has major concern over the use of SCP for human beings because of the following reasons: 1) The high nucleic content of the SCP may elevate serum uric acid level, which may result in kidney stone formation or gout. 2) Certain skin reactions may occur by consuming foreign proteins 3) Possibility of carrying over of carcinogenic factors 4) possibility of gastrointestinal reactions.

Production of SCP

a. **Production of SCP by algae:** Algae can be grown photo synthetically or autotrophically in the Presence of sunlight, artificial light or heterotrophically in dark with organic carbon and energy sources.

Microorganisms

The most important algal strains used for SCP production include:

Chlorella sorokiniana, Scenedesmus acutus, S. quardicanda, S. obliqus, Spirulina maxima, S. platensis.

Growth conditions

In an open circulating system particularly sewage oxidation ponds, mixed culture of algae tend to predominate rather than a single strain. The limiting factor in the algae growth on a large scale is illumination. Carbon dioxide is the source of carbon for photosynthetic-autotrophically growing cultures. Air contains only 0.03% carbon dioxide. Accordingly, additional carbon dioxide must be supplied to the cultures. Some algae can also be grown in lakes rich in sodium carbonate. In sewage ponds, the algal growth is limited by the extent of liberation of carbon dioxide and ammonia by bacterial action. Slow and uniform liberation of carbon dioxide is necessary to provide a uniform supply of carbon source to the growing culture. Nitrogen sources suitable for algal growth include ammonium salts or nitrates. The N-sources together with phosphorous and mineral nutrients may be readily available in sewage ponds. However on other water supplies where synthetic media are used in culturing algae, these nutrients may have to be supplied. Aseptic conditions are not maintained during the large-scale growth of algae in ponds but the potential contamination by pathogenic bacteria may be given serious consideration. The key factors in a large-scale algal cultivation are agitation and flow rates. Agitation prevents sedimentation and keeps the cells in suspension while flow rate adjustments allows the detention period of algal culture to exceed the generation time. This helps in maximum population development.

Cell recovery

Cells must be recovered by concentration, dewatering and drying. The inorganic compounds like Aluminum sulfate, calcium hydroxide and cationic polymers have been investigated as flocculating agents but these do not separate from algae. Algae may auto flocculate in shallow ponds at pH 9.5 or above without flocculants. Ion exchange resins at pH 2.8-3.5 can also recover algae but this method is expensive. Spirulina maxima float on the surface in clumps when maximum growth is attained. It can be harvested by skimming at much lower cost than would be incurred by centrifugation.

SCP production by bacteria and actinomycetes

Bacteria are of interest for use in SCP production because of following reasons: 1) High growth rate (20-30 min. in bacteria, 2-3 hours in yeasts and 16 hours in algae, molds and higher fungi). 2) Bacteria can utilize a variety of carbon and energy sources, renewable sources as carbohydrates (starch, sugars and cellulose) and non-renewable sources as hydrocarbons and petrochemicals. Actinomycetes are of interest because their growth patterns and substrate utilization patterns are similar to bacteria.

Microorganisms

Methylococcus capsulatus, Methylomonas methylovora, Methylophilus methylotrophus, Pseudomonas spp.

Growth conditions

Some important considerations for selecting bacterial cultures suitable for use in SCP production are: High Specific growth rate, yield on a given substrate, pH and temperature tolerance, less aeration requirement, genetic stability and

freedom from associated bacteriophage. Criterion for using bacteria for SCP production is as follows: 1) Strains should not be pathogenic to plants, animals or human beings. 2) Strains should not have potential for mating with known pathogenic bacteria to yield pathogenic hybrids. Growth condition requirements are: 1) Carbon and Nitrogen ration in the medium should be 10:1 or less to favor high protein content in cells and to prevent accumulation of lipids or storage substances such as Poly-β-hydroxy butyrate. Nitrogen is added as anhydrous ammonia or ammonium salts and phosphates as phosphoric acid (feed grade) to avoid contamination with arsenic or fluorides found in crude industrial phosphoric acid. Minerals are added as Magnesium and Manganese in water. Other minerals are added as sulfates and hydroxides but not chlorides. pH is controlled in the range of 5-7. Temperature tolerance is important for bacteria grown on alcohol hydrocarbons. Maintenance of sterile conditions is important to avoid contamination at pH 5-7. Different carbon sources may be used for bacteria these may be carbohydrates sugar, starch, cellulose, and baggase, wheat bran, and wood, petroleum products diesel oil, gas oil, and n-hexane, proteins (Collagen, meat packing waste), methane, Alcohols (methanol) and n-alkanes.

Cell recovery

A number of problems come in way in bacterial cell recovery process because large volume of water is to be handled and bacteria have very small size in addition to that the bacterial cell densities are very close to water. Therefore they are separated by centrifugation, filtration and electrochemical coagulation methods. After separation they are spray dried.

Production of SCP by yeasts

Yeasts are probably the most widely acceptable and used microorganism for SCP production. Yeasts have the capacity to grow on a variety of substrate including waste products that contain pentose sugars. Yeasts in general have several advantages over bacteria and algae these are: 1) better public acceptance 2) lower nucleic acid content 3) easier harvesting because of size and concentration 4) high protein content 5) production of vitamins of B-12 category 6) growth in substrates of low pH.

Growth conditions

a. Raw materials used as substrates: Materials that are employed to produce SCP by yeasts

include: 1) molasses from sugar industry 2) starch after hydrolysis 3) spent sulfite liquor from paper industry 4) acid hydrolysates from wood industry 5) whey from dairy industry 6) hydrolyzed starchy foods (grains and cull potatoes, fruit wastes etc.) 7) methane 8) methanol and ethanol 9) alkanes and paraffins 10) gas oil 11) combustion gas Substrates and nitrogen concentration for yeast growth should be adjusted to provide C □ N in the range of 7: 1 to 10: 1 to favor high protein content. Concentration of carbohydrates in batch culture should range from 1-5% while in continuous culture having hydrocarbons and alcohols; lower concentrations are to be used. Ammonium salts or anhydrous Ammonia is suitable as nitrogen source. Phosphoric acid is used to adjust pH. Yeasts when grow on substrates like carbohydrates, hydrocarbons or alcohols, a lot of heat is liberated therefore heat tolerant varieties should be employed for production (e.g. Hansenula polymorpha can grow at 37-42°C) and cooling water or refrigeration facilities should be employed in the fermentation. The growth rate of yeasts under aerobic conditions depends upon the rate of mass transfer of oxygen and substrate to and across the cell surface. Yeast SCP production may or may not take place under sterile conditions. In either batch or long-term continuous yeast production one must balance the need for contamination control by maintaining sterile conditions, with the capital and operating costs of the equipment required. The temperature of fermentation is maintained at 36-37°C and pH 4.5-6.0. Yields are 45% or more of the dry yeast on the basis of the sugar fed.

Cell recovery

Yeast cells range in size from 5-8µm and have a density 1.04-1.09 gm/cm^3. They can be recovered readily from the medium by centrifugation. The cells are centrifuged in 2 stages. In the first stage it is dewatered and yeast cream is separated. This is followed by two subsequent washing centrifugations. The final washed yeast cream usually contains 15-20% solids. In SCP by *Kluyveromyces fragilis* grown on cheese whey, the entire growth medium is passed through a three-stage evaporation to concentrate the solids from 8-27% to give a feed grade product. The separated cells can either be drum or spray dried.

Ethanol as Fuel

Ethanol can be used as such or in blends with petrol as a motorcar fuel. About 10% ethanol can be used without modifications in the engine but the only disadvantage it has is that it increases the octane rating. Combustion engines can be built to run straight on ethanol or on ethanol of lower concentration (80% ethanol and 20% water). Current interest in various parts of the world centers to use a blend of ethanol called 'Gasohol', which is a blend of 90% unleaded petrol and 10% ethanol. Anhydrous ethanol is preferred for this application but 96.5% ethanol can be used if mutually miscible solvent such as isopropyl alcohol is added.

Raw materials

Forty-five kilograms of fermentable sugars (glucose) is assumed to yield 18-23 kg or 23-28 litres of ethanol. The yield is same for starchy raw materials i.e. between 40-50% based on dry weight of carbohydrates. Complete hydrolysis of starch yields about 50 kg of glucose but conversion is never complete, therefore with 90% conversion the yield is 40-50%. For cellulosic raw materials yield is less because α-cellulose is quite resistant to enzymatic attack.

Sugar containing raw materials

Any sugar containing fruits, fruit juices or extracts, sugar-containing effluents from canneries, sugar beet, sugar cane, sugar cane molasses, cheese whey or sweet sorghum may be used as raw materials.

Starchy raw materials

Cereal grains (wheat, rice, corn etc.), root crops (cassava), tubers (potatoes) are gelatinized by heating and then hydrolyzed to fermentable sugars by enzymes.

Cellulosic raw materials

Saw-mill residues, paper-mill residues, newsprints, potato peelings, rice straw, corn stover, peanut shells, cocoa and coffee husk, tobacco stalks and wheat straw contain α-cellulose, hemicellulose and lignin. These materials when used are delignified, hydrolyzed and then enzymatically treated with cellulases to convert them into fermentable sugars.

Microorganisms

Most of the commercial production is carried out with *Saccharomyces cerevisiae.* For production of ethanol from pentose solutions from corn stover hydrolysis and from spent sulfite liquor of wood industry *Candida utilis* is used and glucose is fermented with immobilized cells of *S. cerevisiae*. Many strains of *S. cerevisiae* readily produce 10-13% ethanol by volume in batch fermentation of molasses solutions with initial fermentable sugars 20-25%. Higher concentration of ethanol can be achieved in Fed-batch culture. The fermentation of whey requires the use of dairy yeast Kluyveromyces fragilis and *K. lactis*.

Nitrogen sources

Assailable Nitrogen sources such as ammonia, ammonium salts, urea, or amino acids are added in addition to phosphorus in the form of ortho-phosphate. Minor quantities of potassium, magnesium, calcium and trace elements are also added. The addition of vitamins is rarely required but thiamine has been often seen to accelerate the rate of fermentation. *S. cerevisiae* requires the presence of biotin. The best source of all required nutrients including the trace elements and vitamins is yeast extract.

Fermentation

Biochemical basis of fermentation: Yeasts, under anaerobic conditions, metabolize glucose to ethanol primarily by way of the EMP pathway. The overall net reaction involves the production of 2 moles each of ethanol, carbon dioxide and ATP per mole of glucose fermented. Therefore on a weight basis, each gram of glucose can theoretically give rise to 0.51g of alcohol. The yield attained in practical fermentations however does not exceed 90-95% of theoretical. This is due to the requirement for some nutrient for to be utilized in the synthesis of new biomass and other cell maintenance related reactions. Side reactions also occur in the fermentation (usually glycerol and succinate), which may consume up to 4-5% of the total substrate.

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ ATP$$ (Gay Lusac's Equation)

Glucose Ethanol carbon dioxide Energy

Modes of fermentation

Batch fermentation: Most of the production of ethanol is carried out on the same lines as Established hundreds of years ago. These methods are based on the simple batch fermentation of substrates inoculated with culture microorganism at temperature 20-30 °C, initial pH adjusted to 4.5 and the time varies between 36-72 hours. Final ethanol concentration is usually 10-16%. Batch technology has been preferred because of its ease of operation, low requirement of sterilization, use of unskilled labor, low risk of financial loss and easy management of feedstocks. It has its disadvantages also such as low productivity due to long turnaround times, expenditure of cooling as fermentation by this mode raises the temperature of the fermentation broth and initial lag in growth.

Continuous fermentation: In continuous fermentation, fresh medium along with inoculum (yeast)is fed to the fermenter and an equal volume of the fermented liquor is withdrawn from the fermenter for the recovery of ethanol and yeast. Continuous ethanol production eliminates much of the unproductive down time associated with the batch fermentation process. The microorganism inoculated in the continuous mode should always be in its exponential growth phase because this increases the time dependent productivity of ethanol. The rate at which medium in the fermenter is added or withdrawn is usually expressed as 'Dilution rate'. It is denoted by a letter 'D'. The time for which the fermentation solution remains inside the fermentor for fermentation is referred as 'Residence Time'. The dilution rate is the ratio of withdrawn liquid to the volume of total liquid in the fermentor. Hence, a dilution rate D = 0.1 indicates that one-tenth of the fermentor liquid is withdrawn per hour (or the residence time in the fermentor is 10 hours). In continuous fermentation residence time and dilution rate should be so adjusted to get higher ethanol production.

Cell recycle: In Cell recycle, yeast cells are separated from the withdrawn fermented solution by means of centrifugation or by any comparable process or settling and returned to the fermentor. This is done to overcome low cell density limitations and to maintain high cell concentration in the fermentor.

Vacuum fermentation: This is a novel fermentation process in which alcohol formed by fermentation is continuously removed from the system so that it does not become toxic to the microorganism. This process was described by Cysewski and Wilke in 1977, 1978 and by Ramalingham and Finn in 1977. The former took the advantage of high volatility of alcohol and conducted the fermentation at

temperature conducive for the growth of yeast and sufficient vacuum to boil off the alcohol while latter used vacuum lesser than what former had used and temperature 30 °C.

Aeration

Aeration is done to keep the cells under suspension and to maintain viability in cells. Therefore sufficient amount of air should be sparged into the fermentor so that dissolved oxygen concentration (expressed as Oxygen Tension) may not exceed its toxic levels.

Distillation

Distillation process may be divided into two stages i.e. Distillation proper and Rectification. In distillation proper, volatile components of the fermentor are separated from the insoluble solids and ethanol is concentrated to a distillate containing 30-96% ethanol by weight. In rectification, ethanol is separated from other volatile components such as aldehydes (acetaldehyde) and fuel oils (amyl alcohol 60-80%, isobutyl alcohol 15-30%, and n-propyl alcohol 0-10%) about 1 litre of acetaldehyde and 5 litre of fuel oil is produced for every 1000 litre of ethanol produced. The amount of the volatile component depends upon the starting material used for fermentation and fermentation process itself.

Ethanol has a lower boiling point than water. Concentration of ethanol is therefore always more in the vapor phase of ethanol solution in equilibrium with the water. When distillation of the water and ethanol mixture is carried out, ethanol concentration of ethanol is always more in the distillate. Water and ethanol form an azeotrope. (Azetrope is a mixture of volatile substances that at a given concentration has identical liquid and vapor compositions). For ethanol and water this concentration is 96% by weight of ethanol and the boiling point of ethanol is 78.2°C. Therefore initially easy separation of ethanol from the broth becomes increasingly difficult as concentration 96% by weight of ethanol is reached because volatilities of both water and ethanol become identical. Boiling at this point gives a vapor of same composition as that of boiling liquid and no further concentration of ethanol can be achieved.

Anhydrous ethanol may be obtained from 96% ethanol solution by forming a ternary azeotrope with benzene. This ternary azeotrope contains 74% benzene, 18.5% ethanol and 7.5% water and its boiling point is 68oC. Since this azeotrope contains more water than the ethanol water azeotrope, it can be distilled overhead and leaves anhydrous ethanol behind. Such a process usually consists of two steps simple fractionation to produce near azeotrope mixture followed by ternary distillation with an added chemical agent yielding anhydrous ethanol. If the extraneous material is less volatile than the feed, it is called a solvent and the operation is called extractive distillation. When the extraneous material is more volatile than the feed it is withdrawn with the overhead product and the operation is called azeotrope distillation. In addition to these methods water may also be removed from ethanol water azeotrope by the use of adsorbents, such as cellulose, or other dry plant materials (cracked grains). Inorganic desiccants have also been suggested for this purpose. An endless loop of yarn fibers is also used to remove water from ethanol water azeotrope.

By-Products of ethanol fermentation

During the fermentation of ethanol many by-products are also formed which must be removed effectively by the distillation system. The by-products more volatile than the ethanol are mainly aldehydes with acetaldehyde the principal compound. Methanol in small amounts is also formed by the hydrolysis of pectins present in the fermentation broth. The less volatile compounds formed as a by-product are Fusel oils which are a mixture of isomers of amyl alcohol 60-80%, isobutyl alcohol 15-30% and n-propyl alcohol 0-10%. Separation of by-products: The aldehydes are removed relatively easily due to the low boiling point of acetaldehyde as a distillation head product; however the separation is not complete. The residual aldehyde content in alcohol is typically 5-100 mg/L. Fuel oil, owing to its complex composition and limited solubility in water is more difficult to separate. Dry fuel oils boil in the range from 128-137°C and normally, being even less volatile than water, they remain in the bottom product. In the presence of water however, the boiling point of the mixture falls below that of the water alone as a consequence of their immiscibility. Fuel oil, therefore rises from the bottom of an alcohol-purifying column and concentrate in the middle of the column, from they are removed. In a continuous process fuel oil is removed by bleeding from 6th to 15th plate of the rectification section and then fed to the fuel oil separator.

Then specification of the industrial alcohol depends upon the intended use. Premium grade alcohol has low water content, low odor ratings and high permanganate time test values (presence of aldehydes).

a. **Definitions**

A. Denatured Spirit- Alcohol that is denatured by adding 10% methanol is called denatured alcohol or denatured spirit.

B. Rectified Spirit- Alcohol from which fuel oil has been removed is called rectified spirit or rectified alcohol.

C. Anhydrous Alcohol- Alcohol that has very less water content is called anhydrous alcohol.

D. Potable Alcohol- Alcohol that can be used for drinking purposes is called potable alcohol.

Different Types of Substrates for Industrial fermentations

Substrates are used as components of media suitable for the growth of microorganisms or the production of desirable microbial metabolites. Nutrient medium form the environment, in which microorganisms grow and from

which they draw substances necessary for the synthesis of cellular components and to produce energy that runs the biochemical machinery of the cell. Bio-chemically substrates may be classified as: 1) Simple-that contain the lowest amount of energy, e.g. water, carbon dioxide, oxygen, nitrogen and inorganic ions, 2) High-energy-contain highest amount of energy and 3) Intermediary substrates-that are formed during the transformation of simple substrates to the high-energy substrates. The function of the nutrient medium is to ensure the growth of the microorganism and has to contain individual substrates in a form best accessible to the microorganism, which usually means in solution. However, in some cases solid media are used and cells from gaseous phase may sometimes utilize some substrates. Besides major components medium also contains many other components necessary for microbial growth. Physico-chemical properties and medium composition adjustment help in the maintenance of the maximum rate and desirable direction of the microbial process.

Complex media are prepared from natural raw materials of animal or plant origin; the exact chemical composition of these media is usually not known and individual components can be determined only with difficulty. A complex medium is prepared by dissolving appropriate natural substances in water and is often further processed by hydrolysis, clarification complemented by additional nutrients, growth factors, and missing trace elements. Synthetic media are prepared by dissolving defined chemicals in distilled water to eliminate completely all minerals and trace elements usually present in un-distilled water. Complex media are most often used for technological purposes while synthetic media are employed only in special cases e.g. in the processing of substrates from the chemical industry or utilization of some waste materials. In laboratories usually synthetic media are used despite their numerous shortcomings because they are defined therefore it is possible to draw exact material and energy balances from changes in the levels of individual components. As a qualitative and quantitative combination of substrates, the medium must meet the microbiologist's demand. The consumption of substrates in the laboratory is very small and it is quite within the range of microbiologist's to use more expansive substrates that cannot be utilized on commercial scale due to certain reasons. According to state of the medium, media can be classified as: 1) Solid media. 2) Liquid media. 3) Gaseous media. The solid media are present in the solid state and they are used immediately on dissolving in distilled water. The liquid media are readily utilized as they contain more water. These can be converted onto solid ones or gels by the addition of certain thickening agents like agar-agar, gums, and gelatin etc. The gases also play an important role in the growth and multiplication of the organisms and they also have a profound effect on the production of metabolites. The main components of a medium are as follows: 1) Water. 2) Carbon source. 3) Nitrogen source. 4) Accessory substances. 5) Antifoam agents.

Water

Water is an indispensable component of a medium. It is much needed by microorganisms for fermentation process. It is also needed in other processes like isolation of the cells and products. In fermentation, we usually use tap water for the preparation of culture media, separation and washing of microorganisms. The quality of water used depends upon the process in which is used e.g. A water of low grade quality can be used for cooling purposes, whereas for the preparation of media and isolation of cells and products etc. a water of high quality i.e. Potable water or treated water is needed. The treated water can be produced by four ways: 1) By adjusting the chemical composition i.e. by removing salts or ions like iron, free chloride or chlorine, salts of carbonic acid and adjusting the hardness. 2) By removing the extraneous materials by sedimentation, filtration etc. 3) by removing microorganisms from the water i.e. by the processes like filtration, sterilization and disinfection. 4) By removing the colloids i.e. by the process of osmosis. The quality of water always fluctuates and can be controlled by various chemical and biological methods e.g. when levels of ammonia in water increases, the water is said to be polluted and has to be treated by various chemical methods or by growing certain organisms like ammonifying bacteria. Similarly when levels of salts of nitrous acid or nitric acid rise above the normal limits, growing denitrifying bacteria also controls them. When iron levels in water increases, it causes discoloration of microorganisms such as yeasts and inhibit the biosynthesis of various substances e.g. antibiotics. It may also produce corrosion like effect in water. The norms for high quality water are 20-100 germs per ml. The hardness of water is due to the presence of various salts of calcium and magnesium, which when rise above their normal limits, they lead to instability in product yields. In that case, water is deionized and appropriate portions of other salts are added. Such types of treatments are only possible in laboratory and at commercial scale; the cost of applicability becomes very high. The quality of ground water now days is further deteriorating due to the increases use of chemicals in agriculture and utilization of nuclear materials in the warfare. So the demand of water for the commercial purpose has further increases and further prospects of research in this regard are needed.

Carbon sources

They represent the main component of nutrient media quantitatively. They are utilized for the biosynthesis of cell materials and are also used as main energy source. Carbon is also used to build carbohydrates, lipids, fats and proteins etc. in the cell and it is derived from various organic compounds added in the medium. Some new types of substrates have also come into light such as synthetic alcohols, alkanes and many hydrocarbons. The nature of microbial process, product purity and metabolism of the inoculum microorganism may dictate the use of chemically defined carbon sources such as glucose, sucrose, lactose or starch as well as complex raw materials like molasses, sulfite liquors, wood hydrolysates, cellulose and raw materials of petrochemical origin.

Carbohydrates

They form a distinct group of organic compounds that are divided into three categories as monosaccharides, disaccharides and polysaccharides.

Monosaccharides

Glucose-In fermentation process, glucose is the most frequently used monosaccharide (Grapes contain 25-30% of glucose) but it has its own disadvantages like Catabolite repression and Pasteur effect. For industrial purposes glucose is obtained by the hydrolysis of starch both by chemical and enzymatic methods. Since it is rapidly assimilated therefore a continuous inflow of glucose is required in growing cultures for the biosynthesis of complex compounds. It is sterilized separately from the other components of the medium because at neutral pH and at high temperature it undergoes Maillard's reaction with amino acids to give dark brown colored solution. It is usually sterilized at pH 3.0 as a 30-50% solution (w/v); under these conditions the solution remains colorless. In microbial processes, the so-called "Hydrol", a waste product in the manufacture of pure glucose, can also be used as a substrate.

Disaccharides and oligosaccharides

1. Sucrose- It is obtained from sugar cane or sugar beet and is available in different levels of purity. It is much needed for the fermentation by fungi, yeast and in the fermentation of microorganisms those biosynthesize antibiotics, amino acids and organic acids.
2. Lactose- Lactose is present in the milk of mammals at the levels of 3-8% and is prepared by the evaporation of whey that remains after the processing of milk to butter. Only a few species of microorganisms can assimilate lactose; yeasts with a few exceptions do not assimilate lactose. *Penicillium chrysogenum* can assimilate lactose in low but in efficient levels. The main disadvantage of the lactose is its varying quality, which can be attributed to different milk processing operations.

Polysaccharides

These substances represent a suitable source for bacteria, yeast and fungi and are used in the biosynthesis of many products. They are macromolecular, forming colloidal solutions or exhibiting negligible solubility in water, for this reason they must often be converted into a soluble form before being used in nutrient media.

Starch: It is one of the most important polysaccharide both biologically and industrially. It is accumulated as a reserve substance in fruits, seeds, and bulbs of higher plants in the form of granule of different shapes and sizes. It is usually denoted according to its origin i.e. according to the plant from which it is manufactured i.e. Potato starch from potatoes, Maize starch from maize, Wheat starch from wheat, Rye starch from rye and Barley starch from barley. Starch granules are hygroscopic and do not changes at low temperatures while at high temperatures, they swell and change into starch gel. The gelation is usually performed at 100 °C in water. At 120-130 °C, the gel liquefies and may be easily degraded by enzymes. Starch is a mixture of two related polysaccharides, amylose (linearly arranged chain of α-glucosidically linked molecules of D-glucose with 1-4 bonds) and amylopectin (branched chain of D-glucose molecules bonded also -glucosidically by 1-4 and less often 1-6 bonds); one molecule contains more than one thousand glucose residue. Acid hydrolysis of amylose yields first maltose and then glucose. Enzymic degradation by amylases proceeds via amylodextrin and low molecular weight dextrins to maltose.

Cellulose: It is a structural polysaccharide, forms a substantial part of the plant cells and is found abundantly in nature. Isolation of pure cellulose is difficult because other natural substances like lignin, hemicellulose and waxes accompany it. For industrial purposes, cellulose is obtained from wood and straw of various plants. Recently an intensive research has been initiated concerning cellulose-degrading enzymes like cellulases. The cellulase is a multienzyme complex of three enzymes namely endoglucanase, exoglucanase and α-glucosidase that act synergistically to form glucose. A compound known as "Cadoxen" (25-30% ethylene diammine in water and 4.5-5.2% cadmium) has the capacity to disrupt the crystalline structure of cellulose, which renders it susceptible to rapid and total hydrolysis to form soluble products. Other agricultural wastes, which can be used as carbon sources are: Molasses from sugar industry, Barley water and its products from brewing industry, sulfite waste liquor from paper and pulp industry, acid-wood hydrolysates from wood and paper industry, vegetable oils and animal fat from oil industry and alcohol and hydrocarbons from petrochemical industry.

Molasses: It is a thick, syrup-like, viscous and dark colored liquid obtained as a byproduct in the production of raw and refined sugar from sugar cane or sugar beet (remains after the crystallization of main fraction). It is named after its source as Cane or Beet molasses. Cane molasses contains a high level of sugars

Barley and its products: Barley and its products such as malt, wort and malt extract. Malt is prepared by the germination of barley. During malting amylases and pectinases are only partially activated since the process takes place at low temperatures and in a medium containing only vegetable liquor. In the brewery malt is crushed, mixed with water and kept at a higher temperature; under these conditions the enzyme reactions proceed rapidly and completely as far as the degradation of malt starch is concerned. Wort is obtained from malt residue by filtration. It is used as a raw material for Beer brewing. After the addition of hops it is boiled and filtered. The resulting solution is hopped wort; after cooling it is inoculated by a culture of brewer's yeast and fermented. Malt extract is prepared from wort by filtration and evaporation; it is thick syrup containing 80% dry weight that may be vacuum dried to a powder.

Sulfite waste liquor-It is a waste product in the production of pulp from wood by the sulfite method. It is a brown-yellow liquid with a density of 5 to 90 B at 20°C. Its specific weight is 1.045 to 1.060. It contains 8 to 14% dry weight that consists of 18 to 20% minerals and 80 to 90% organic substances. The inorganic part is composed chiefly of sulfur dioxide, sulfite and calcium sulfate. A part of the calcium is bound to lignin as a calcium lignosulfate salt, which is water soluble in acidic media. Other inorganic compounds present in the liquor are salts of heavy metals such as copper, arsenic and lead. These heavy metals ions in their minute amount also affect the growth of microorganisms. The organic compounds found in the liquor i.e. lignin and carbohydrates, are released from wood during treatment with the sulfite cooking acid. A short cooking at low temperature yields solid pulp containing higher levels of hemicelluloses and lignin; therefore the resulting liquor contains lower amount of these substances. On the other hand long and intensive cooking at higher temperature produces soft pulp having very less lignin and hemicelluloses resulting in liquor of higher contents of these substances. The liquor further contains small amounts of volatile acids like acetic and formic acid. Further fermentation of sulfite liquor depends upon how much carbohydrates are released during cooking and pulping. The amount of carbohydrates varies according to the species of the pressed wood and technology of pulping. The composition differs in liquors from conifers and from deciduous trees. The total carbohydrates in the coniferous wood liquor usually include 30-40% mannose, 30-35% glucose, 10% galactose, 15% xylose, and 5-10% arabinose. In deciduous trees, the liquor contains 50% xylose, 15-20% arabinose, 10% methylpentoses, 10% mannose, and about 10% uronic acids. In addition they contain higher levels of furfural, which originates from pentoses during cooking at high temperature and pressure. The furfural affects negatively on the growth of microorganisms. The sulfite waste liquor cannot be used directly for fermentation in the form obtained from the cooker since 1) it contains a considerable amount of sulfur dioxide, which has to be removed. 2) Has fluctuating pH values, which can be adjusted with calcium carbonate or calcium hydroxide. 3) it may contain insignificant levels of assimilable nitrogen and phosphorus and these nutrients must therefore be added. Sulfite liquors are used especially for the production of ethanol and production of fodder yeasts.

Acid wood hydrolysates: The resulting pulp after the sulfite waste liquor has been removed still contains a considerable amount of carbohydrates other than cellulose. These are removed by low concentrations of acids, which hydrolyse hemicelluloses to soluble sugars; this process is called as prehydrolysis. Prehydrolysis removes only hemicelluloses and not cellulose in contrast to the complete hydrolysis that cleaves all the polysaccharides including cellulose. The prehydrolysis is carried out on deciduous wood, cereals, rape or rice straw, and reed grass. The straw prehydrolysates contain higher amount of pentoses and therefore are used for the production of fodder yeasts. Vegetable oils and Animal Fats-Majority of the vegetable oils are easily metabolized by microorganisms therefore they are used as a carbon sources in many microbial processes especially in the production of antibiotics. They are also often used as an antifoam agent. Fats on the other hand are solid at room temperature and their liquefaction requires high temperature. For this reason they are not so often used as substrates.

Alcohols-Synthetic alcohols, especially ethanol and methanol have importance as potential raw materials for microbial processes. Ethanol is available in sufficient quantities, has a reasonable price, standard quality and homogeneity. Its physico-chemical properties place it among surface-active substances; hence it increases the foaming of nutrient media. However, the foam is thin and facilitates a better distribution of air into liquid. Synthetic alcohol is easy to produce in pure form and is highly water-soluble. Its disadvantage is the presence microorganism inhibiting substances like crotonaldehyde. Methanol is also water-soluble and has similar properties like that of ethanol. In comparison with ethanol it has a higher volatility and toxicity. Hydrocarbons-Microorganisms can utilize almost all hydrocarbons to larger or some smaller extent. Bacteria can assimilate all hydrocarbons while yeasts can assimilate only some of them. The best carbon sources among hydrocarbons are n-alkanes. Alkanes can be divided in to several groups according to the number of carbon atoms they contain. These are: C1- C9 n-alkanes, C10-C20 n-alkanes and C20 and higher n-alkanes. C1-C9 n-alkanes-Methane is the most widely used alkane in this group because it is readily available. It is assimilated by most of the bacteria but not readily by yeasts. An amount of 1 Kg of methane yields 0.8 kg of dry cell matter. Due to their yield and narrow range of possible sources the alkanes of this group are not suitable for microbial industry. C10-C20 n-alkanes -The alkanes of this group are readily assimilated by almost all microorganisms including bacteria, yeasts, actinomycetes and molds. The yield of cell mass per consumed alkane of this group is 90-100%. C20 and higher alkanes-Assimilation of this group of alkanes is slower because alkanes of this group do not exist in liquid state at normal cultural temperatures.

Nitrogen sources

Substances containing nitrogen in various forms may serve as sources of nitrogen for microbial growth. The range of nitrogen assimilation is much wider than the carbon assimilation. Many microorganisms, especially lower fungi, utilize nitrogen from inorganic nitrogen sources like inorganic ammonium salts. Ammonium sulphate, the cheapest of these compounds is often used in combination with ammonium hydroxide and gaseous ammonia for continuous regulation of pH. Another very good source of nitrogen is urea; since it decomposes at high temperature is should be sterilized by filtration. Quality wise nitrogen

sources cannot match with the pure laboratory chemicals because of the economic reasons. Although many microorganisms assimilate inorganic nitrogen, they often grow faster in the presence of organic nitrogen sources. At present many suitable nitrogen sources of plant and animal origin are available as waste products.

Inorganic and synthetic organic nitrogen sources

Ammonium sulphate is readily soluble in water; the purest form used in microbial industry is technical ammonium sulphate that contains a minimum of 20.7% nitrogen. The so-called coke and gas sulphates are considerably contaminated with tar substances and are used only exceptionally as substrates. Diammonium hydrogen phosphate, which is used especially in yeast production, is a source of both nitrogen and phosphorus. Ammonia is used either in the form of 25% aqueous solution or in gaseous form. Urea is used only exceptionally in microbial industry i.e. in the production of fine and high-grade products, due to its relatively high price.

Natural sources of nitrogen

Corn Steep Liquor-It is obtained as byproduct in the production of maize starch. It contains biologically active substances that differ qualitatively and quantitatively according to the quality of the corn used and its technological processing. These substances are released into the corn steep liquor during corn steeping and some microbial processes, especially lactic acid fermentation participating in the release, occur during steeping process. Moyer and Coghil first used corn steep liquor in the Penicillin fermentation in 1946 and that enhanced the yield of antibiotic many folds. Corn steep liquor contains nitrogen substances, lactic acid, reducing substances and ashes with many trace elements. An important criterion of the quality of corn steep liquor is the ratio of amino nitrogen to total nitrogen, which should be about 0.4 i.e. approximately 2% of amino nitrogen dry matter. This ratio indicates the intensity of lactic fermentation during steeping. Another criterion is the content of amino acids, which should be higher than 20%.

Potato liquor and fermented extracts from bran and oil seeds-Sometimes liquor from potato starch industry and fermented extracts from bran and oil seeds are used as substrates in microbial industry. Potato liquor is obtained as follows: The liquid formed by pressing potato pulp in starch production is subjected to sulphuriation and lactic acid fermentation by Lactobacillus delbrueckii and carefully dried. Though it contains many reducing substances yet it lacks amino acids like arginine, glutamic acid, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, proline, threonine and valine. The fermented extract of bran and oil seeds is prepared as follows: Wheat bran and ground tobacco seeds are mixed with water and a small amount of lactic acid and superphosphate is added. After six days of lactic acid fermentation at 50oC the liquid is filtered and evaporated in vacuum. Yeast extract-It is available either dry or in the form of paste. The extract is prepared by autolysis or plasmolysis of Baker's or Brewer's yeast. To remove bitter hop substances, Brewer's yeast is washed at pH 6.5 to 7.0 prior to treatment. Yeast cells must be kept in a viable state during washing and filtration in order to prevent losses of cell components. Baker's yeast does not need washings. The autolysis is performed in a large vessel under continuous stirring at a temperature above 75°C. The yeast is then transferred to a vessel containing sodium chloride solution, which induces cell plasmolysis. It is then filtered or centrifuged to separate cell walls and membranes. The liquid is rapidly concentrated at 37oC to prevent degradation of thermolabile substances and also to prevent contamination. Drying the liquid form in an atomizer or spontaneously in vacuum makes the powered form of the yeast extract. Yeast extract is a mixture of amino acids, peptides, water, soluble vitamins and carbohydrates. Soybean and Peanut flour-Soybean flour is an important source of nitrogen. Its most important constituents are proteins 44-47%, fat 5.5-6.0% and phosphorus 0.62-0.65%. It is obtained by the extraction of oil from soybeans. Peanut flour is also obtained after the extraction of oil from peanuts. It's important constituents are nitrogen, fat and phosphorus. The composition of proteins is variable and depends on the source of raw material. Both peanut and soybean flours are used as substrates in the biosynthesis of antibiotics. Peanut flour is suitable for the biosynthesis of Penicillin and Lysine while Soybean flour is suitable for the biosynthesis of Chlortetracycline. Peanut flour is not suitable for the biosynthesis of Tetracyclines.

Antifoam agents

Media when are rich in natural substances; foaming occurs in the microbial processes. This foam poses difficulties when it is uncontrolled. The outflow of a large amount of foam from the fermentor may reduce the volume of the culture and may lead to contamination. Various antifoam devices and certain natural or synthetic antifoam agents are employed to control foam in a fermentation system. The effect of these substances is based on their ability to reduce the surface tension of the liquids. The term 'Antifoam' is used for the substances that are added before the foaming actually occurs in fermentation while the term 'Defoam' applies to a substance added afterwards to knock down the foam. The natural antifoam agents used are soya, rape, coconut, sunflower and mustard oils. Antifoam agents of animal origin are tallow and deodorized fish fat. Mineral oils may also be used. Another group of substances used are alcohols. The most common compound of this group is Octadecanol either pure or in combination with lard oil or mineral oil. Specially designed antifoam agents are also available; among them are silicon oils. The silicon oils are usually used in water based emulsions containing 10% silicon oil. They are most effective in bacterial or yeast cultures but are less effective in cultures of filamentous microorganisms. Since the microorganism does not assimilate silicon oils, that is why silicon oils are very efficient

and a single dose at the start of the fermentation is usually sufficient to prevent the formation of the foam. A similar effect is exhibited by polyalcohol's with molecular weight of 2000 and alkylated glycols. Phosphorus and magnesium are important compounds of nutrient agar medium since they participate in energy transduction, especially in reactions mediated by ATP. Microorganisms need calcium, potassium, sulfur and sodium; therefore are added to the medium for microorganisms. Though trace elements such as iron, cobalt, copper and zinc are indispensable, they are usually present as impurities in other components and in water. Enrichment by these trace elements is necessary only when analysis of synthetic media or raw materials indicate their shortage. A major element in media is phosphorus, which can be added both in organic and inorganic form. The compound most frequently used in industrial microbiology is Diammonium hydrogen phosphate that also serves as nitrogen source. Superphosphate is used in the production yeast; it is a mixture of calcium hydrogen phosphate and calcium sulphate with variable content. Growth Factors and Vitamins Growth factors and vitamins are usually present in natural sources of carbon or nitrogen compounds. When these natural substrates are used as the only source of vitamins, it becomes sometimes necessary to add additional growth factors and vitamins in a pure form. Vitamins of the B-complex category are necessary as growth factor in the production of Baker's yeast. The production of amino acids by auxotrophic mutants requires the presence of certain vitamins; shortage or excess of these substances in the medium affects the yield. Production of certain secondary substances is greatly increased when precursors of these substances are added into the medium e.g. when phenylacetic acid or phenylacetamide is added into the medium; biosynthesis of Penicillin G increases but when phenoxyacetic acid is added into the medium; biosynthesis of Penicillin V is enhanced.

Yogurt

Yogurt is defined as the end product of a controlled fermentation of high solids whole milk with a symbiotic mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in the ratio of 1:1 for the manufacture of high-grade yogurt. Among the various cultured dairy products it is unique because it is the only product in which acetaldehyde in relatively high concentration is desired as an essential flavor component. Similar sour milk products are called as 'Matzoon' in Armenia, Matsoni in Georgia, Leben in Syria, Egypt and Algeria and Matsurad or Jodda in Sycilly.

Forms of yogurt

At present yogurt is sold and used in various forms in different parts of the world. These forms are as follows: 1) Plain Yogurt (Set type and Stirred type). 2) Flavored yogurt without fruits (lemon or Vanilla). 3) Yogurt with fruit puree (French style or Swiss style or Continental style -Whole or sliced fruits are mixed uniformly throughout the yogurt and Sundae style -Fruits may be filled in the bottom of the cup or on top of the yogurt to be mixed by the consumer). 4) Soft frozen yogurt (Flavored and Unflavored). 5) Hard frozen yogurt (Flavored and Unflavored). 5) Frozen yogurt sticks. 6) Fluid yogurt drinks. 7) Fruit topped with yogurt 8) Spray dried yogurt products for confectionary, bakery and soups.

Yogurt ingredients

a. Dairy ingredients: The dairy ingredients for the preparation of yogurt include whole, partially defatted milk, condensed skim milk, cream, and non-fat dry milk. All the dairy ingredients should be of very high bacteriological quality. Mastitis milk, rancid milk, partially fermented milk; milk containing antibiotics and sanitizing chemical residues cannot be used for yogurt production. Since yogurt is a manufactured product, it may have variations from the quality standards established by the marketing considerations. Therefore it becomes necessary to standardize the milk used in the yogurt production. The milk fat levels in yogurt range from 1.0 -3.25%. Yogurt can be classified on the basis of fat present as: 1) Yogurt -contains minimum of 3.25% fat, 2) Low-fat yogurt -containing not less than 0.5% and not more than 2.0% milk fat, 3) Non-fat yogurt -contains less than 0.5% milk fat. In all the categories of yogurt, a minimum milk solid non-fat and minimum titrable acidity stipulated is 8.25% and 0.5% respectively. In order to standardize the milk solids non-fat, cream, partially skimmed milk, and skim milk alone or in combination, concentrated skim milk, non-fat dry milk, or other milk derived ingredients may be used. The milk-derived ingredients include casein, sodium and calcium caseinates, whey; whey protein concentrates alone or in combination.

Sweeteners

Sucrose is the major sweetener used in yogurt production. Sometimes corn sweeteners and honey may also be used. The level of sucrose in yogurt mix appears to affect the production of lactic acid and flavor in the yogurt. Sucrose concentrations above 4% inhibit the growth of *S. thermophilus*. Sucrose may be added in a dry, granulated, free-flowing, crystalline form or as a liquid of 67^0Brix. Commercial yogurt has an average of 4.06% lactose, 1.85% galactose, 0.05% glucose and pH of 4.15. In frozen yogurt 6% corn syrup solids are added. Non-nutritive sweeteners like calcium-saccharine, maltol, and sorbitol alone or in combination may be used for diabetics. Sometimes lactase is added to break the lactose of milk.

Stabilizers

They produce smoothness, body texture, gel structure, reduce wheying off or syneresis, and increase shelf life. Stabilizers function through their ability to form gel structure in water, thereby leaving less free water for syneresis. In addition, some stabilizers perform their action by forming a complex with casein. A good stabilizer should not impart any flavor, should be effective at low pH values, and should be easily dispersed in the normal working temperatures in a dairy plant. The stabilizers generally used are gelatin,

vegetable gums like carboxy methylcellulose, locust bean, carob, guar and seaweed gums like alginates and carrageen an. Sometimes agar-agar or pectin is also used. Calcium chloride is added to control whey separation.

Fruit preparations for flavoring yogurt

Fruit preparations are present at the level of 15-20% in the final product. Generally fruits are added to meet the market demand. In every type of flavored yogurt, the composition of basic fruit preserve remains the same while its pouring styles are different. Fruit preserve consists of 55% sugar and a minimum of 45% fruit which is cooked until the final soluble solid content is 68% or higher (65% in the case of certain fruits). Frozen fruits and juices are the usual raw materials. Commercial pectin, 150grade, is normally utilized at a level of 0.5% in preserves and the pH is adjusted to 3.0-3.5 with a food grade acid, viz. citric acid during manufacturing of the preserve. Calcium chloride and certain food grade phosphates are also used in several fruit preparations. In Fruit on Bottom or Eastern Sundae style yogurt, 59ml fruit preserve on bottom is followed by 177ml of inoculated yogurt mix on the top. No flavor or sweetener is added. It is incubated till pH becomes 4.2 and then refrigerated. In western Sundae style yogurt, the top yogurt layer contains flavors and colors while in Swiss style yogurt, yogurt and mixed fruit layers are mixed together.

Flavors and colors

Only certified flavors and colors should be used for the preparation of yogurt. It should meet the following requirements. It should 1) exhibit true color and flavor of the fruit when blended with yogurt, 2) be easily dispersible in yogurt without causing texture defects, phase separation or syneresis.

Microorganisms

The culture is specified as a mixture of Lactobacillus bulgaricus and Streptococcus thermophilus in 1:1 ratio. The inoculum culture can be regenerated from a lyophilized culture tubes on media containing milk as a component.

Microbiology of yogurt

Streptococcus thermophilus is commonly referred as coccus and *Lactobacillus bulgaricus* as rod. Equal numbers of both of these bacteria are desirable for the production of tartness and green flavor of yogurt. The fermentation is carried out within a temperature range of 35 -45°C, optimum being 40°C. During fermentation, both rods and cocci work together to produce yogurt. Rod shaped Lactobacillus bulgaricus grows first slowly but their weak proteolytic activity liberates sufficient amount of the amino acids (glycine and histidine) and peptides to stimulate cocci. Streptococcus thermophilus now grows vigorously to produce moderate amounts of lactic acid, acetic acid, acetaldehyde, diacetyl and formic acid. When the pH of the yogurt mix (initial 6.3-6.5) drops below 5.5, the rapid growth of cocci is arrested and that of rods is favored. Oxidation-Reduction potential of the system is depressed. Depletion of oxygen and the availability of the formate ion stimulate the growth of rods. Until the pH value reaches 4.2 Streptococci grow very slowly. Below pH 4.2 Lactobacilli grow rapidly and dominate the fermentation. The major portion of the lactic acid and acetaldehyde necessary for the characteristic flavor of yogurt is contributed by the lactobacilli but the second part of the fermentation is greatly aided by the initial metabolic activity of the coccus component. Yogurt should be cooled at pH value of 4.2-4.3 to obtain a high-grade product.

Flavors of yogurt

Typical flavor of yogurt can only be detected in plain yogurt because it is a product of bacterial symbiosis. The production of flavor depends upon the proportion of rods and cocci and their combined metabolic activity. The distinctive flavor is contributed by lactic acid (odorless) and by acetic acid, which are volatile and have strong odors. Milk components and other ingredients in the mix also play a role in providing a background flavor. To obtain a good flavor one should keep in mind that 1) starter strain should be selected with a view to obtain a good symbiosis 2) proportion of rods to cocci should be equal 3) rapid cooling should be done at pH 4.2. If the pH value falls below 4.2, in large tanks that require a long time to cool down to 10oC then the desired cocci to rods ratio and flavor balance is lost. The product so obtained will be too sour and coarse.

Characteristics of a good yogurt

1) The body of yogurt should possess a relatively high viscosity and should be firm and cohesive enough to be removed and eaten with a spoon. 2) There should be very little wheying off during normal handling for mixing, cooling, pumping and packaging. 3) It should have a smooth, rich texture free from lumps, granules or graininess. 4) There should be no gas packets, fissures or gassy effervescence.

Factors affecting body characteristics of yogurt

The factors are: 1) Fat and SNF concentration in the mix. 2) Stabilizers used. 3) Control over weighing and blending of ingredients in the mix. 4) Heat treatment of the mix 5) Concentration of protein by new process such as ultrafiltration. 6) Concentration of calcium and magnesium ions. 7) Type of starter culture used 8) Incubation conditions used. 9) Initial pH value of the mix. 10) Handling during pre and post incubation operation. 11) Sucrose concentration in the mix.

a. Defects and their causes in yogurt

1) Graininess -If coagulum becomes exceedingly firm before stirring, the finished yogurt tends to grainy. In addition, the use of rennet to obtain a good body invariably leads to graininess. 2) Coarse Texture -Disturbance of the yogurt mix just before the gelling stage gives rise to coarse texture. 3) Granular feeling in Mouth -Inadequate mixing of the powdered products causes a granular feeling in the mouth. 4) Gas packets and Fissures -These are caused by

the trapped carbon dioxide or hydrogen gas produced by contaminant flora like E. coli, Bacillus spp. and yeasts. 5) Slimy feeling in Mouth -Slime producing strains when used in starter culture would result in a mouth feeling similar to the white of eggs. 6) Off Taste and Off Odor -These develop due to faulty fermentation.

Kefir

It is a historic and old product from Caucasus region of Russia. Kefir grains are used as starter culture that can be reused several times. The kefir grains are gelatinous white or cream-colored irregular granules from the size of wheat grain to walnut. These are made up of a polysaccharide called 'Kefiran', which is associated with denatured milk protein. These are insoluble in water and swell up when soaked in water to form a jelly like product. Bacteria and yeasts are present within the folds of the grains and there may be some symbiotic relationship between the grains and microorganisms. Various kinds of microorganisms are involved in the kefir fermentation these include: *Saccharomyces kefir, Torula kefir, Lactobacilli, Streptococci, and Leuconostoc spp. Coliforms, micrococci* and spore forming rods contaminate the fermentation.

Method of preparation

Fresh cow's milk is pasteurized at 85 °C for 30 minutes. It is inoculated with kefir grains taken from the previous batch after cooling to 25 °C. The incubation at 23 °C yields a soft curd, which after being agitated forms a beer like foam and fuzziness from which kefir grains come upward with the evolution of carbon dioxide. Kefir grains are separated and washed in cold water. They are stored in cold water at 4 °C or are dried in a warm oven. The kefir grains have alcoholic, yeasty sour with tangy effervescent flavor. Fresh kefir grains have activity up to 8-10 days while dried grains may remain active for 8-12 months. They contain lactic acid 0.8%, alcohol 1%, carbon dioxide and flavor compounds (acetaldehydes, diacetyl and acetone).

Acidophilus Milk or Reform Yogurt

It is a product formed by fermenting milk with an authentic culture of *Lactobacillus acidophilus*. The organism has its unique features that it can survive in the severe conditions of intestinal tract of human beings, animals and birds. Its ability to initiate growth in, or form colonies on media containing bile salts should be used as a distinguishing characteristic. The strains of man and animals have certain differences like DNA of human isolates generally had lower G+C ratio than that of pig and chicken biotypes. The acidophilus milk has its therapeutic value in controlling intestinal disorders but its mode of action has not yet been elucidated.

Method of preparation

The acidophilus milk is an extremely sour product. The finished product contains very little, if any metabolic byproduct other than lactic acid. It is prepared from partially skimmed milk, which is heated at 120°C for 15 minutes. The heating at such high temperature denatures the proteins of the milk that releases some peptides required for the growth of bacteria. It is then cooled to 37-38°C and inoculated with 5% milk starter culture. The incubation at 37-38°C for 18-24 hours yields Acidophilus milk, which is quickly cooled to 5-7°C and bottled when final acidity of the finished product reaches 1.0%.

Bulgaricus Butter Milk Or Bulgarian Milk

It is prepared from cow or mare's milk, which is fermented by a pure culture starter of Lactobacillus bulgaricus. This is particularly consumed in various parts of the world particularly Balkan countries. The starter produces the required acidity and flavor. It can also be prepared from cow or buffalo's skimmed milk.

Method of preparation

The milk is heated at 95°C for 30 minutes and cooled to 37-38°C. It is then inoculated with 2% milk starter culture prepared with Lactobacillus bulgaricus. The milk is incubated until the acidity reaches 1.4%. The product is cooled to 7°C. After incubation a curd like smooth mass is formed that is diluted and churned. The butter is removed. Buttermilk left has its acidity in terms of lactic acid (0.25%). The final product lacks aroma but has a pleasant flavor. It can be used as a substituent for milk but it has very less sugar content. It has diuretic effect when taken in large quantities **(Flow Chart 3)**.

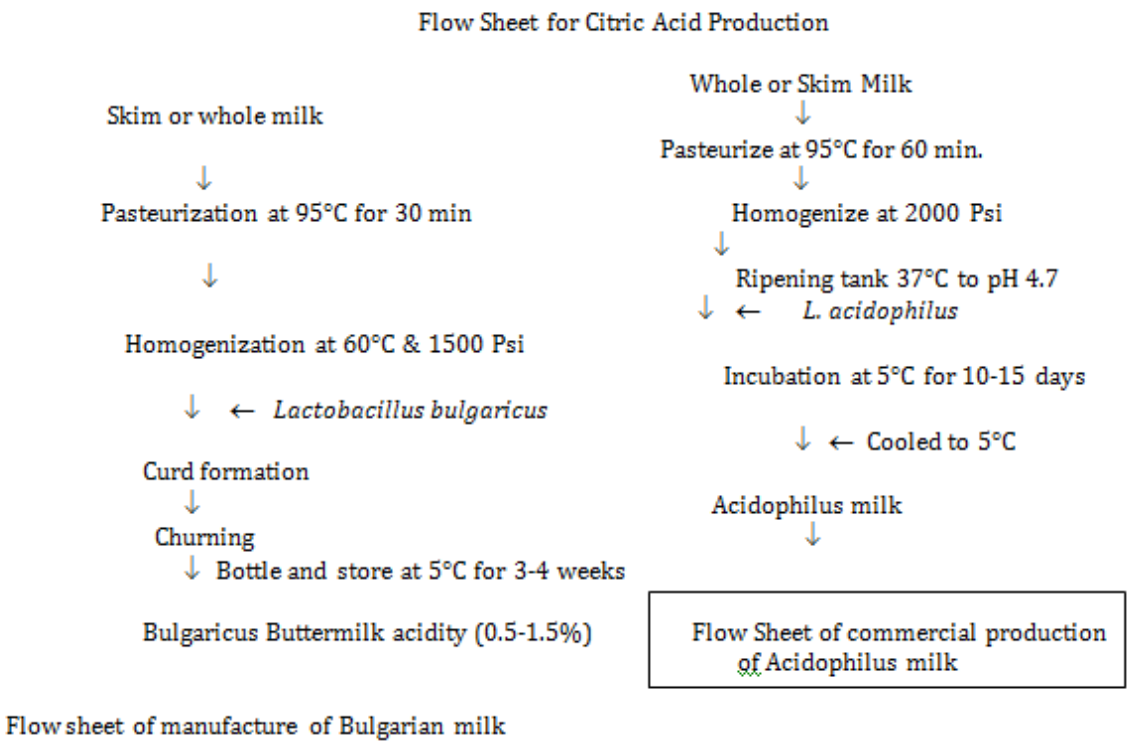
Role of heat treatment at high temperature in culture dairy products

Heat treatment at 85-95 °C for 30 minutes or equivalent is an important step in the manufacture of cultured dairy products. The heat treatment 1) produces a relatively sterile medium for the exclusive growth of starter culture. 2) Removes air from the medium to produce more conducive medium for microaerophilic lactic cultures to grow. 3) Effects thermal breakdown of milk constituents, especially proteins, releasing peptones, sulfhydryl groups which provide nutrition and anaerobic conditions for the starter. 4) denatures and coagulates milk albumins and globulins that enhance the viscosity and produce custard like consistency in the product.

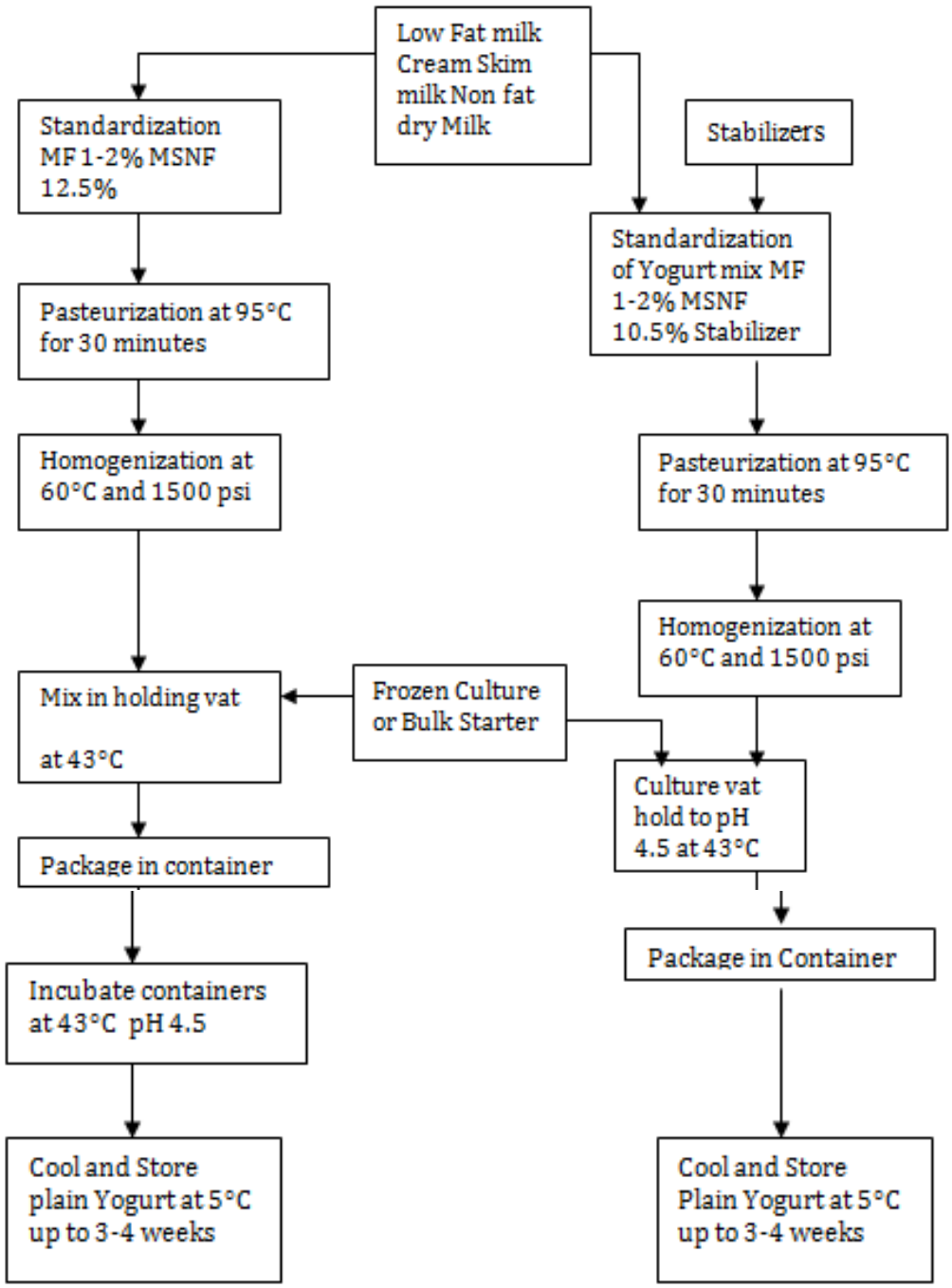
Flavor production in cultured dairy products

The characteristic flavor of cultured dairy products is produced by the activity of lactic culture by metabolic transformation of milk constituents. An important transformation of milk components into an essential flavor compound in cultured dairy products involves citrate metabolism. Milk contains an average of 0.2% citrate and it exhibits the greatest seasonal fluctuation (0.07-0.4%). Citrate is converted into 'Diacetyl' by flavor bacteria *Leuconostoc cremoris and Streptococcus lactis sub sp. Diacetylactis*. *Leuconostocs* ferment citrate only when there is sufficient acid development during the fermentation. Because leuconostocs do not produce much acid from the

lactose of the milk, therefore require an associative growth with lactic acid producing strain. *Streptococcus lactis sub sp. diacetylactis* on the other hand produces sufficient lactic acid, which required for the fermentation of citrate to produce diacetyls. The literature contains conflicting reports on the biosynthetic pathways involved for the production of diacetyls in aroma bacteria. Diacetyl synthesized by flavor bacteria in dairy products does not accumulate indefinitely. Once the concentration of diacetyl precursor, citrate falls below a critical value, the diketone is rapidly converted into a flavorless compound, Acetone. An enzyme called diacetyl reductase is widely distributed among flavor bacteria and other contaminating psychotropic bacteria commonly found in dairy environment, catalyzes conversion of diacetyl into acetone. Acetaldehyde is another important flavor compound but in cultured creamy butter, cultured buttermilk and cultured sour cream it is undesirable because it imparts a flavor defect referred to as green or yogurt flavor. It is a very important flavor component in yogurt and related products. It is primarily derived from lactose although other mechanisms for production of carbonyl residue are found among lactic acid bacteria. For example, metabolism of threonine and deoxyribonucleic acid results in acetaldehyde formation by bacteria. Many bacteria produce acetaldehyde; major ones are streptococcus lactis subsp. diacetylactis, Streptococcus thermophilus, Lactobacillus bulgaricus. Lactic Streptococci also produce a variety of carbonyl compounds that impart flavor important ones are: volatile fatty acids, formic acid, acetic acid and propionic acid. In koumiss and Kefir alcohol is an important component characteristic of the product. Torula kefir yeast and *Saccharomyces kefir* are responsible for the production of alcohol. One very important compound causing flavor problems in cultured milks is 3-methylbutanal. This imparts a malty flavor to cultured milks and ripened cream butter. Carbon dioxide plays an essential role in the flavor impact of cultured buttermilk, kefir and koumiss. The gas entrapped in the thickened milk provides lift, fizz, or effervescence to these cultured products. Carbon dioxide is derived from lactose by heterolactic bacteria. Fermentation of citrate by aroma bacteria also produces considerable amount of carbon dioxide. The minor metabolic products, although found in small or even trace amounts, may be important in maintaining a desirable flavor balance in cultured dairy products. Any shift in the flavor balance in cultured dairy products may result in the organoleptic perception of off-flavor. Rancid flavor present in milk is derived from straight chain free fatty acids up to 18-carbons by the action of lipase enzyme. Cream flavor is derived from isomeric 18-carbon unsaturated fatty acids by auto oxidation, 4-cis-Heptenol is produced. Butter flavor is due to β-lactones derived from hydroxy fatty acids by ring closure. Sharp sour flavor is produced by the fermentation of lactose to lactic acid by starter culture. Aldehydes, ketones, alcohols and esters combined to give milk a cowy flavor. Acetic acid contributes significant culture flavors. **(Flow chart 4, 5)**

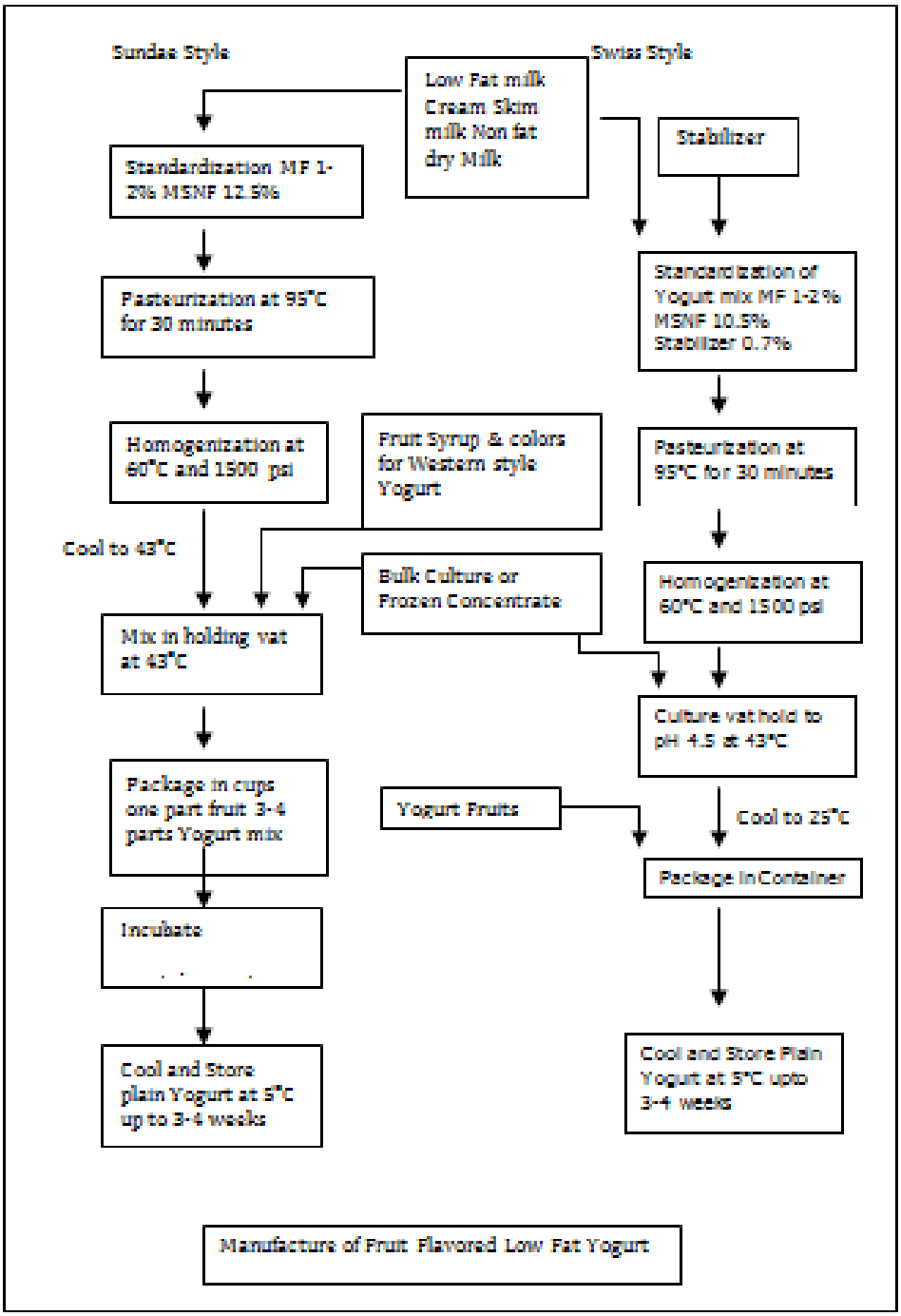


Flow Chart 3



Manufacture of SET and STIRRED TYPES of Plain Low Fat Yogurt

Flow Chart 4



Flow Chart 5

Starter cultures

Cultures used to start the fermentation in the preparation of butter, ripening of cream, souring of milk in cheese manufacture; preparation of various fermented milks and preparation of fermented cereal products are called “Starter Cultures”. They contain microorganisms that produce lactic acid from lactose, citric acid from various compounds or are involved in the fermentation of sugars to produce alcohols, diacetyls, or other flavor and aroma compounds. The microorganisms of the starters may grow in association or in succession to produce the desired product. Various purposes for which they may be used are:

Aroma production

Aroma producing microorganism is Streptococcus lactis that usually grows in fermented milk products. It produces lactic acid from lactose and also ferments citric acid to produce acetic acid, carbon dioxide, acetyl methyl carbinol, diacetyls and 2,3-butylene glycol.

Butter production

During butter ripening Streptococcus lactis produces lactic acid, volatile acids as acetic acid, propionic acid and carbon dioxide. Some neutral 4-carbon compounds like diacetyls, acetyl methyl carbinols and 2,3-butylene glycol add to the flavor and aroma.

Batter fermentation

In case of idli, dosa, jalebi, vada and bhatura, culture of the previous batch is added as starter culture that starts the fermentation. The starter causes leavening and acidification of the batter that produces a pleasant flavor in the batter.

Preparation of fermented milk products

In fermented milk products preparation, the flavor and body is so related with the starter that without good starter culture, the product is sure to become unsatisfactory.

Dahi or curd

Dahi is the most important sour milk product used throughout India. Dahi is the common term used in India for any kind of sour milk. But sour milk from different parts of India varies very much in quality, consistency and microflora. Dahi of Northern India is much thicker than in Southern India where dahi is generally used in diluted form. In some parts of India Cane sugar is added to milk to prepare sweet dahi. Sweet dahi is liked very much in Northern parts of India. Dahi sold in the market is prepared from fresh raw milk, but it is advisable to prepare dahi from boiled milk. Dahi prepared from fresh raw milk is fermented by natural flora i.e. no starter is added. When it is prepared from boiled milk, a little portion of dahi from the previous day is added and milk is allowed to curdle at room temperature. As dahi is prepared without special care regarding its mother starter, its quality varies from place to place and is never uniform.

Method of preparation of dahi: Dahi of good quality should be prepared in the following way: Good

quality whole milk or skim milk may be used for the purpose. Milk should be heated to 85 to 95 °C for half an hour or boiled for 10 minutes. It is then cooled to 45 °C. A small portion of the starter from good quality dahi is then inoculated into the milk. The amount of starter added will vary with the type of the starter, condition of the milk and incubation period. The milk is then incubated at temperature near about 40 ^{0}C. After 24 hours of incubation dahi of good quality may be obtained.

Microflora of dahi: Very little information is available regarding the microflora of this important

milk product. The samples of dahi from south India showed predominance of Lactobacilli. But samples of dahi from North India showed predominance of Streptococci. The species of lactic acid bacteria occurring most commonly in dahi include *L. bulgaricus, Streptococcus thermophilus*, *S. faecalis, S. lactis, L. casei and L. plantarum*, in order of their frequency.

Soy sauce: Soy sauce is a dark brown liquid with a salty taste and distinct aroma, which is made by

fermenting soybeans, wheat, and salt with a mixture of mold, yeast and bacteria. It is a seasoning agent used as a substitute for salt in the preparation of food as well as a table condiment. It enhances the flavor of the food and adds color into meats, sea foods, vegetables etc. The fermentation of soy sauce is essentially a process of enzymatic hydrolysis of proteins, carbohydrates and other constituents of soybeans and wheat to peptides, amino acids, sugars, alcohols acids and other low-molecular compounds by the enzymes of microorganisms. The brewing of soy sauce originated in China many centuries ago and later was introduced in Japan and other oriental countries. Soy sauce is consumed in almost every oriental country and has different names in different countries. It is known as Chiang-yu in China, Shoyu in Japan, Kecap in Indonesia, Kangjang in Korea, Toyo in Philippines and See-iew in Thailand. Japan leads the soy sauce industry in the world. It has not only the largest fermentation plants but also has the most advanced technology. Japanese shoyu is produced primarily in the areas near Tokyo and in Chiba prefecture. Japanese shoyu is mainly of three types: Koikuchi, Usukuchi and Tamari. Nearly 90% of the Japanese shoyu is of Koikuchi type, with dark reddish brown color and strong flavor and made from a nearly equal mixture of soybeans and wheat using Aspergillus oryzae hydrolytic enzymes followed by vigorous lactic acid and alcoholic yeast fermentations. It is pasteurized at a relatively high temperature. Ten percent of shoyu is Usukuchi type, light in color with maximum total nitrogen content 1.2%. Third type of shoyu is Tamari in which major portion is soybean and smaller portion is wheat. The highest quality shoyu is made by fermentation but second and third grades contain some portion of chemical hydrolysates.

Preparation of soy sauce: Soy sauce is prepared by fermentation of a mixture of soybeans and

Cereal usually wheat and salt. However, in recent years, defatted soybean meals and flakes have taken place. Today, more than 90% of soy sauce production in Japan is from defatted soybean products. In addition to the fermentation process, a chemical process in which acid hydrolyzes the proteins and carbohydrates is also being used in some western countries. In this method acid hydrolysis usually results in a complete breakdown of the substrate than enzyme hydrolysis. However, acid hydrolysis cannot perform many other specific reactions or interactions of hydrolyzed products as carried out by the multiple enzyme system produced by molds, yeasts and bacteria. That is

why chemically hydrolyzed product does not possess the flavor and odor of the soy sauce prepared by fermentation process.

Treatments of the raw materials

Soaking soybeans: In preparation for fermentation, selected soybeans are cleaned by ashing and soaked for 10-15 hours at room temperature. The water is changed every few hours to prevent acidification by bacteria. Weight of beans should increase 2.1-2.5 times during soaking.

Cooking soybeans: Hydrated soybeans are cooked for 1 hour in steam at 10-14 lb/sq in. ina rotary cooker (capacity 1 ton). They are then cooled rapidly. When defatted soybean meals or flakes are used, they are first moistened by spraying with water amounting to about 130% of soybean weight and then are steamed at 13lb/sq in. for 45 minutes.

Roasting wheat: Whole wheat or wheat flour is essential for production of typical Japanese

shoyu flavor. Usually low protein flour is used. Wheat is roasted in sand for several minutes at 170-180 °C and then the grains are crushed into 4-5 pieces in a roller mill. The roasting process adds flavor and color to the resulting soy sauce and in addition destroys surface microorganisms and facilitates enzymatic hydrolysis.

Effect of addition of wheat to the fermentation mixture: According to Yokotsuka (1964),

the addition of wheat to the fermentation mixture serves several functions. Firstly, the mold grows better and produces more enzymes on a mixture of wheat and soybeans than on wheat or soybeans alone. Secondly, the addition of roasted, crushed wheat to the cooked soybeans would minimize the growth of undesirable bacteria i.e. moisture of cooked soybeans is 60%, which is ideal for bacterial growth whereas moisture of soybeans and wheat mixture (1:1) is about 45%, which is adequate for mold growth but not for bacteria. Thirdly, wheat serves as a precursor of sugars, alcohols, organic acids, and flavor compounds. Lastly, wheat is rich in glutamic acid.

Addition of salt: Commercial salt is generally preferred for making soy sauce because it may carry an inoculum of halophilic and halotolerant bacteria and yeasts. Salt is added in such quantities that it prevents spoilage and/or food poisoning bacteria and permits the development of flavor and aroma forming bacteria and yeasts.

Role of salt: In addition to giving a salty taste, sodium chloride acts as a preservative and also has a selective action on microorganisms that grow in the fermentation substrate.

Koji and tane koji

Koji is a Japanese name given to a preparation consisting of mold growth on cooked cereals and/or soybeans. Koji serves as an enzyme source for converting complex plant constituents to simpler compounds. Koji is prepared by adding a koji starter culture called “Tane Koji” in Japan in cooked cereals and /or soybean substrate. Different types of tane koji (soy sauce koji, miso koji) are available for commercial use in making soy sauce, miso and others.

Preparation of tane koji: Tane koji is prepared by using naturally selected or mutant strains of *Aspergillus oryzae or A. soyae* to give desirable starter for a particular fermentation. Although strains used for preparation of koji starter are different, the method for preparation is similar. Polished rice is soaked in water overnight, drained, steamed for 1 hour and mixed thoroughly with 2% wood ash as a source of trace elements. The mixture is then inoculated with spores of selected strains of A. oryzae and spread out on trays in layers approximately 1.5 cm deep. And covered with a moistened cloth to favor the growth of mold mycelium. After incubation at 30 °C for 5 days, the rice is well covered with mycelium and with green to yellowish green spores of A. oryzae. The spores are harvested, dried at 50 °C and stored at 15 °C. A koji starter is usually composed of a blend of spores of different strains in a definite proportion, so that various enzymes are produced in proper amounts during the preparation of koji.

Preparation of soy sauce koji: Soy sauce koji is prepared from a mixture of roasted wheat and steamed soybeans inoculated with a koji starter (soy sauce Tane Koji) consisting of selected strains of Aspergillus oryzae grown on polished rice. Inoculated soybean and wheat mixture is placed in wooden or stainless steel porous trays of depth 30-40 cm and several meters in length and width. The trays are then incubated at 25-35 °C. Aeration and moisture is carefully controlled. This step is completed in 45 hours because this prevents the development of Mucor spp. and bacteria and enhances the development of proteolytic enzymes. The end product is called Soy sauce koji, which is a mixture of fungal hydrolytic enzymes on soybean wheat mixture substrate. Soy sauce koji of superior quality has a dark green color, pleasant aroma, sweet but bitter taste and high protease and amylase activity.

Fermentation

The soy sauce koji is mixed with 1.2-1.5 volumes of salt brine (23% w/v salt) to make mash called “Moromi” in Japan. Moromi is fermented in large concrete tanks or wooden vats for 8-12 months. Since koji is not prepared under aseptic conditions, one would expect the presence of yeasts and bacteria in moromi. However pure cultures of Pediococcus soyae, Saccharomyces rouxii and Torulopsis spp. are added to the mash at the start of fermentation and at one month after the start of fermentation to accelerate the fermentation and to improve the flavor of the final product. High salt content ensures the development of flavor enhancing yeasts while lower brine to koji ration result in decreased utilization of total nitrogen. Traditional fermentation starts in April and takes a year to complete. In general low temperature fermentation gives better results, because the rate of enzyme inactivation is slow and enzymes remain active for a longer time.

Pressing: The matured moromi is pressed in a hydraulic press at 100kg/sq cm. (1379lb) for 2-3days into a liquid part, known as raw soy sauce, and a solid cake. When whole soybeans are used as raw materials, soy sauce oil, consisting chiefly of ethyl esters of higher fatty acids, is produced during fermentation and appears at the upper layer of raw soy sauce. This oily layer must be removed and has no potential use. This is one of the reasons that defatted soybean meal is used instead of whole soybeans as raw materials in soy sauce fermentation.

Pasteurization and bottling: The raw soy sauce liquid is pasteurized at 70-80 °C in a kettle or heat exchanger, cooled, filtered to remove precipitates and stored. The final product is bottled for market. The bottles are usually made of plastic or glass and sometimes benzoic acid or propyl or butyl ester of p-hydroxy benzoic acid is added as preservative.

Benefits of pasteurization

1) Flavor, color and clarity is produced by the removal of oil particles mixed with heat coaguable substances 2) Inactivation of enzymes occur, which gives a stable product 3) Concentration of compounds like aldehydes, acetals, phenolic compounds, mercaptans, organic acids, pyrazines, furfurals, and β-diketones is increased 4) Because of the increase in phenolic compounds and organic acids resistance to spoilage by film forming yeasts occur.

Role of Mold in foods fermented by molds

Synthesis of enzymes: Molds, in these food fermentations synthesize enzymes that decompose complex compounds, including proteins, carbohydrates and fats into smaller molecules. At the same time, other compounds may be synthesized from the food substrates. These complex changes are accompanied by changes in the original properties of the raw materials. Taste, flavor, texture, color, palatability and other properties of the raw materials are usually modified in such a way that product becomes more attractive to the consumer. In addition to this general function of producing enzyme, in certain products the mold has a special role to play.

Mold growth: Mold growth on certain products contributes to the appearance of the food, which is desired by the consumer. *Neurospora spp*. provides oncom (fermented food) cake with a coating of its pink orange colored and powdery conidia. Rhizopus oligosporus covers tempeh cake with a clean white mycelium surface layer and additionally has the function of binding together the soybean into a solid, compact cake.

Synthesis of coloring compounds: The function of *Monascus purpureus* during fermentation of Angkak (fermented food) is the production of red colored compound monascorubin and a yellow pigment monascoflavin in soaked rice.

Protection of the product: Molds, which are traditionally used for the fermentation of oriental foods, have shown that they do not produce Toxins. But, on the other hand they resist accumulation of certain toxins which otherwise will be produced by other microorganisms in the food. This could be considered as a protection of the product against other harmful microorganisms. A good example of such a protective role is demonstrated by Rhizopus oligosporus, the mold species used for the production of Tempeh. This mold species does not produce aflatoxins. On the contrary, if aflatoxins are already present in the growth substrate, *R.oligosporus* could lower its contents to about 40% of its original content. In addition it was found that R. oligosporus inhibits growth, sporulation, and aflatoxin production by Aspergillus flavus.

Mold starters

The mold species that are traditionally used for fermentation of foods in different parts of the world belong to different genera. Species *of Rhizopus, Mucor, and Aspergillus* are used for the fermentation of foods throughout the orient, with the exception of Japan. In Japan, it is restricted to species of Aspergillus including A. oryzae and A. soyae. These species are used in Tane koji, which is used as starter culture for the preparation of soy sauce, miso etc. Tane koji is prepared by growing the mold on steamed rice. In other Asian countries, Ragi type starter cultures are in common use. Cultivating molds on cakes made of rice or wheat flour, which has not been steamed or cooked, makes these starters. The difference in preparing growth substrates for manufacture of two types of inocula is thought to be the cause of a natural selection of mold species which are developing in each of the type starters over many centuries, when they were produced with non-aseptic, traditional methods.

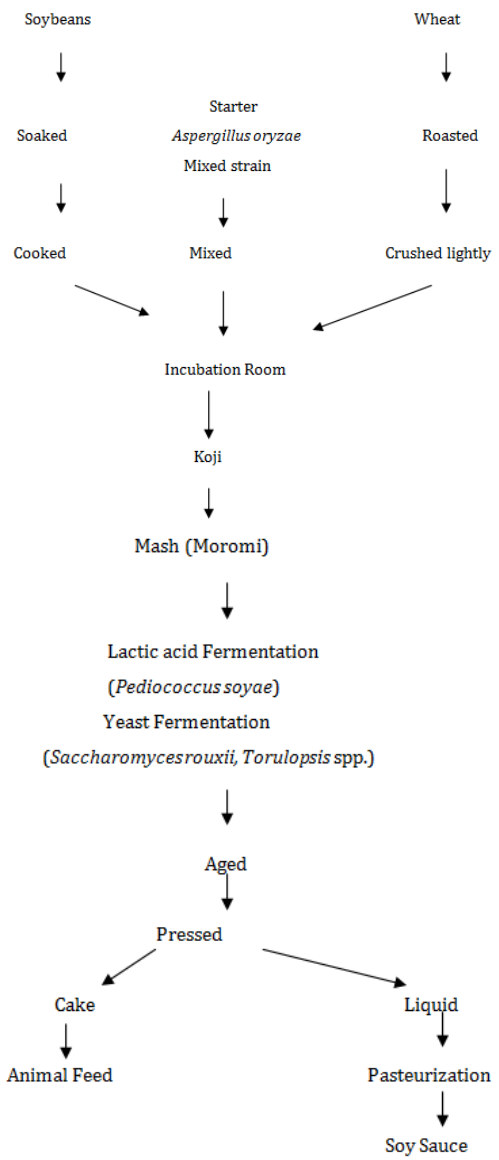
Yeast fermentations

When yeasts are involved in the fermentation process, production of alcohols improves the aroma of the product. In addition, alcohol at a certain concentration makes the substrate unsuitable for microorganisms, which may create undesirable properties in the product. Combined with the organic acids that are produced by lactic acid bacteria, the inhibitory effect of alcohol on undesirable microorganisms is increased. **(Flow Chart 6)**

Miso

Miso is a paste like product made by fermenting cereals, soybeans and salt with molds, yeasts and bacteria in Japan. This product is generally known as Bean Paste. It has the consistency of peanut butter, some smooth and some chunky and its color varies from light yellow to reddish brown. It has distinctive pleasant aroma resembling that of soy sauce, and it is typically salty (degree of saltiness may vary) and may sometimes has sweet taste. It is like soy sauce is used as a flavoring agent in cooking as well as table condiment and can be used in place of soy sauce. The product is known as Chiang in China, Doenjang in Korea,

Tao-Chico in Thailand, and Tauco in Indonesia. All mean bean paste. In Japan, there are many types of miso and they can be prepared by varying the ratios of soybeans to cereals, salt content, length of fermentation and addition of other ingredients such as hot pepper, which is very popular in China and Korea.



Flow Chart 6

Japanese miso can be categorized into three major groups as follows:

1) Rice miso -it is prepared from rice, soybeans, and salt 2) Barley miso -it is prepared from barley, soybeans and salt 3) Soybean miso -it is prepared from soybeans and salt. Each group can further be subdivided into white, light yellow, red according to color, and sweet, medium salty, and salty according to taste. Similar classifications can also be made according to the length of fermentation and fermentation temperature. This can be understood from the following table: (Table 1)

General method of preparation of Japanese rice miso

Cooking Soybeans: Whole soybeans are generally used for the preparation of miso, but sometimes dehulled soybeans or full fat soybean grits are also used for making white or yellow rice miso. Soybeans used should be of large size because the ratio of hull to cotyledons is lower in large sized beans. They should have high water absorbing capacity and when cooked under described conditions, the beans should be homogeneously soft with fine texture and bright color. To prepare the whole soybeans for fermentations, they are washed, soaked in water for about 20 hours at 16 °C and drained. The soaked beans are then cooked in water (white miso) or steamed at a temperature 11 °C for about 20 minutes in a closed cooker and slightly mashed **Table 2**.

Preparation of rice miso koji

For Rice miso koji preparation, polished rice is used. Since it is essential that the mold mycelium quickly penetrates the rice kernels, the rice is soaked in water (15°C) overnight or until moisture content is about 35%. Excess water is drained off, and the soaked rice is steamed at atmospheric pressure for 40 minutes. After cooling to 35°C, tane koji (koji starter culture), which is a blended mixture of several different strains of A. oryzae, prepared as described in the soy sauce preparation, is sprayed over the rice and mixed well. The inoculated rice is then incubated in a temperature and humidity controlled room. In about 40 -48 hours at 30 -35°C. The rice is completely covered with white mycelium of the inoculate culture. Harvesting is done while koji is white and before any sporulation has occurred. At this time, koji has a pleasant odor, lacks any musty or moldy odors, and is quite sweet in taste. The koji is removed from the fermentor and mixed well with salt to stop any further development of the mold.

Yeast and bacterial fermentation

Next fermentation is carried out under anaerobic conditions by yeast and bacteria. Cooked and slightly mashed soybeans are mixed with the salted koji and inoculated with a starter culture containing pure culture of Yeasts (Saccharomyces rouxii, Torulopsis spp.) and bacteria (Pediococcus halophilus, Sterptococcus faecalis). Miso from the previous batch can also be used as an inoculum of yeast and bacteria. Sufficient water is added to bring the moisture content equal to 48%. The mixture, now known as Green Miso, is thoroughly blended and tightly packed into a vat or tank for fermentation at 25 – 30 °C. During the fermentation period, the green miso is transferred from one vat to another at least twice to improve fermentation conditions. Fermentation time varies widely depending upon the type of miso. It is one week for white miso, 1 -3 months for salty miso and one year for soybean miso.

Table 1

Soybean : Rice : Salt	Type	Color	Taste	Fermentation Time	Fermentation Temperature
100 : 200 : 35	White miso	Bright light yellow	Sweet	2-4 days	50 °C
100 : 60 : 45	Salty miso	Light yellow	Salty	30 days	30 –35 °C
100 : 50 : 48	Red salty miso	Yellow red	Salty	60days	30-35 °C

Table 2

Protein	Isoelectric point	Protein	Isoelectric point
Egg albumin	~ 4.6	γ_1-Globulin	6.6
Haemoglobin	6.8	Lysozyme	11.0
Pepsin	~ 1.0	Myoglobin	7.0
Serum Albumin	4.9	Chymotrypsinogen	9.5
β- Lactoglobulin	5.2	--	--

Aging and packaging

At the end of fermentation, the fermented mass is kept at room temperature for about 2 weeks to ripen. The aged product is then blended mashed, pasteurized (60 – 70°C for 30 minutes), and packaged. Traditionally, miso is sold in wooden kegs of various sizes. Presently, it is sold in sealed polythene bags or tubes. For packaging into the plastic bags, miso must first be pasteurized at 60 –70°C for 30 minutes to prevent swelling. Sorbic acid or its potassium salt is also added at a level of less than 1 gm/kg of miso **(Flow Chart 7)**.

Dehydrated miso powder

This product has become increasingly popular. The dehydration is carried out by freeze-drying process. Dehydration process does not affect the flavor of the product. It has its potential use as an ingredient in the instant mix products**. (Flow chart 7)**

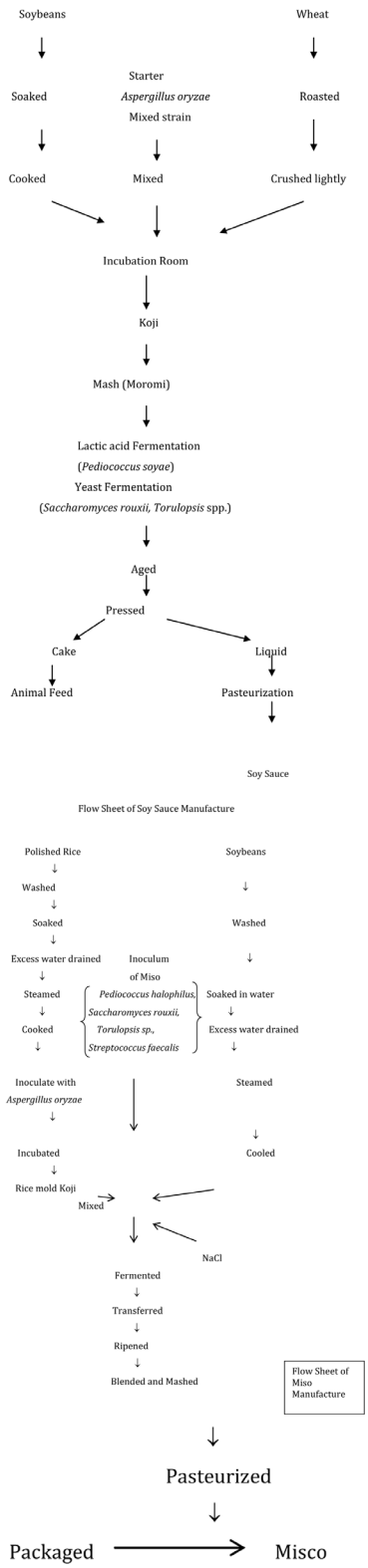
Tempeh

Tempeh or Tempe kedelee is a cake -like product that originated in Indonesia and is widely consumed in the regions of Malaysia and Indonesia. It is prepared by fermenting dehulled and briefly cooked soybeans with a mold, Rhizopus; the mycelia bind the soybean cotyledons together in a firm cake. The raw tempeh has a clean, fresh, and yeasty odor. When sliced and deep -fat fried, it has a nutty flavor, pleasant aroma, and texture that are familiar and highly acceptable to almost all people around the world. Unlike most of other fermented soybean products that are used as flavoring agents or relishes, tempeh is used as a main dish and meat substitute in Indonesia. It is easy to cook and does not possess the beany flavor of soybeans that most people find unpleasant. Because of its high protein content and universally acceptable taste and texture, it can be a potential source of low -cost proteins.

Preparation of tempeh

Microorganism: The mold used for tempeh fermentation was reported earlier to be Rhizopus oryzaebut in Indonesia, a strain identified as Rhizopus oligosporus saito NRRL 2710 is considered better producer of a good product. This strain is characterized by sporangiospores showing no striations and being very irregular in shape under any condition of growth. The sporangiospores are short unbranched and arise opposite to rhizoids that are much reduced in length and branching. It's all isolates also show Chlamydospores. Rhizopus oligosporus is highly proteolytic, which is important in tempeh fermentation because of the high protein content of the substrate. Two proteolytic enzyme systems were observed in the fungus; one has an optimum pH at 3.0 while the other at 5.5. Both the enzyme systems have maximum activities at 50 – 55 °C and are fairly stable at pH 3 -6. They rapidly denature at pH below 2 or above 7. In addition to high protease activity, the mold possesses strong lipase activity, low amylase and no detectable pectinase activity.

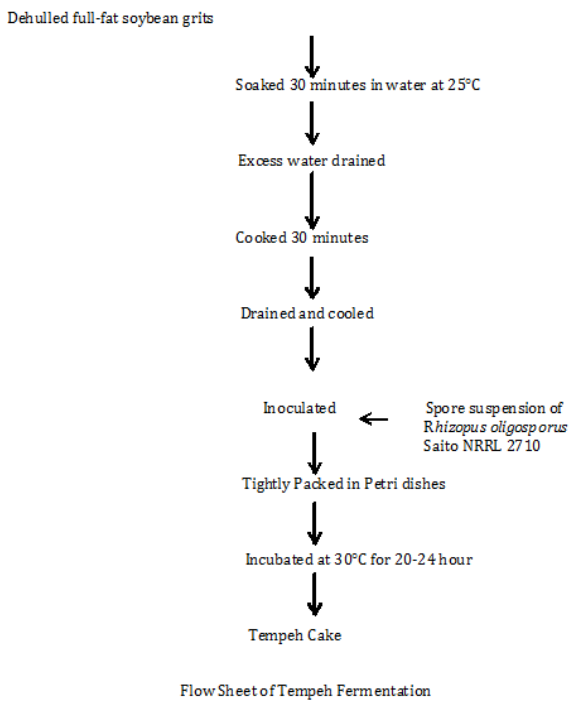
Preparation of soybeans for fermentation: The Full -fat soybeans are soaked in water overnight at room temperature. The soybean cotyledons can be mechanically cracked into 4-5 pieces, so that they absorb water easily and this also reduces the soaking time from 20 hours to 30 minutes. They are dehulled by hand or by a simple roller mill. The grits of beans are boiled in water for 30 minutes. They are then drained and spread to cool and surface drying.



Flow Chart 7

Fermentation

A pure culture of *R. oligosporus* spore suspension (prepared by adding a few milliliters of sterilized distilled water to the slant culture) already grown on potato dextrose agar and incubated at 28 °C for 5-7 days is mixed with the cooled and surface dried soybeans at the rate 106 spores/100gm of cooked soybeans. The inoculated soybeans are tightly packed into an appropriate container (petri dish) for incubation to obtain a final product in which a white mycelium developed abundantly but black spores are minimal. Rhizopus oligosporus like many molds require air to grow, but it does not require as much aeration as many other molds. In fact, too much aeration causes spore formation and may dry the beans resulting in poor growth. Therefore, it is important to properly pack the beans for fermentation. Many workers have successfully tried different types of packing materials. These are petri plates, aluminum foils or metal trays, with perforated or woven mesh bottoms and covers or perforated plastic film covers or perforated plastic bags and tubings. When the fermentation is complete, the beans are covered with and bound together by white mycelia. Thus raw tempeh looks like a firm white cake and has an attractive and slightly yeasty odor. Prolonged fermentation often causes the product to become obnoxious due to the enzymatic breakdown of proteins **(Flow Chart 8)**.



Flow Chart 8

Idli

Idli is a breakfast food in most parts of India especially popular in southern India. It is acidified and leavened through fermentation by hetero fermentative acid bacteria rather than by the activity of the yeast. It is closely related to sourdough bread of the western world, but it does not depend upon wheat or rye as a source of protein to retain the carbon dioxide gas during leavening. The importance of idli lies in 1) its high degree of acceptability as a food 2) its protection against food poisoning and transmission of pathogenic microorganisms, because of acidity and 3) the fact that idli fermentation can be used in many parts of the world using various combinations of cereal grains and legumes to produce acid, leavened bread, or pancake like products 4) No wheat or rye flour is needed. Idli is a small, white, acid leavened, and steamed cake made by the bacterial fermentation of a thick batter made from carefully washed rice (Oryza sativa) and dehulled black gram dhal *Phaseolus mungo*. Idli cakes are soft, moist and spongy and have a desirable sour flavor. It is served like a pancake with butter, honey, and jam or with other sauces. It can also be consumed directly "out-of-hand" following steaming or the cake may be deliciously flavored with fried mustard seeds and chopped coriander leaves. The unflavored cakes are eaten with chutney and /or samber, a thin-spiced soup of dhal and vegetables.

Details of manufacture of Idli

Ingredients: Idli preparation contains a number of different ingredients these are 1) Rice 2) Black gram dhal 3) Salt 4) water. Dehulled soybeans or Bengal gram can be used as a substitute for black gram dhal and a number of cereal grains can replace rice. However, there may be marked change in the texture and flavor when using substituted materials. It has been reported that rice variety and its physical characteristics are very important to produce a good quality idli. White Kar and IR20 varieties of rice have given much better performance in the production of idli, especially the White Kar variety because of its high amylose content, low amylopectin content better gelatinization, and better water uptake ability.

Proportion of cereal to legume

Ordinary idli consists of three parts rice and one part black gram dhal plus salt to taste. Kancheepuram idli is prepared from one part rice and one part black gram dhal plus cashew nuts, ghee, salt, pepper, ginger and cumin added to taste. Normally proportions of rice to black gram dhal varies from 4:1 to 1:4, the 2:1 being the best. It has been seen that when black gram dhal proportion is less than 25%, the steamed idli was hard and organoleptically unacceptable whereas when it is more than 50%, the product obtained is too sticky to be acceptable. Thus, not only can the ingredients be varied, but the proportions can also be varied within a wide range and still an acceptable product is obtained.

Soaking

Generally, the ingredients are soaked separately in water at room temperature for 5 to 10 hours before grinding to prepare the batter. Parboiled rice semolina can frequently be used while dry black gram dhal flour has been found unsuitable for the preparation of idli. If the idli batter is to be made without inoculation, it is essential that cereal and legume be soaked, ground with water and incubated at room temperature. Hot soak or hot grind will destroy the organisms essential for fermentation and, unless they are replaced by an inoculum, the fermentation will not proceed properly.

Proportion of water and salt to other ingredients

The amount of water added to the rice and dhal batter has varied from 1.5 to 2.2 times the dry weight of the ingredients. Batter should be rather thick for idli. Generally salt 0.8 to 1% is added to the batter as a seasoning before fermentation.

Fermentation time

Fermentation time varies from 14-24 hours, with overnight being the traditional time interval for the preparation of idli. The fermentation time must be sufficient to allow a definite leavening of the batter and allow for the development of pleasant acid flavor.

Inoculum

Ordinarily, the microorganisms developing during initial soak and then during the overnight fermentation are sufficient to leaven idli. The Central food technological Research Institute recommends adding one tablespoonful of buttermilk to each pound of its dry idli mix. The microorganisms that develop during overnight soaking are: *Leuconostoc mesenteroides, Streptococcus faecalis, Pediococcus cerevisiae, Lactobacillus fermentum, Torulopsis candida, Trichosporon pullutans, Torulopsis holmii* etc.

Incubation temperature

Ordinarily, the idli fermentation is carried out at room temperature. It generally means that temperature of 25 °C to 30 °C is probably an optimum temperature.

Steaming

The fermented batter is steamed as soon as the product has become leavened and acidified for 15-30 minutes at atmospheric pressure to get a soft, spongy and sour tasted and flavored idli. The fermented batter is poured into the cups of an idli steamer, which is placed in a covered pan and steamed until the starch is gelatinized and idli cakes are soft and spongy. They are generally consumed on the same day and there is no effort to preserve the product. The acid content of the product retards the growth of food poisoning and food spoilage causing microorganisms.

Microbiology of fermentation

In a study of sequence of microorganisms that developed during soaking of ingredients and subsequently during fermentation of the batter at 30 °C, it has been found that Leuconostoc mesenteroides and Streptococcus faecalis developed concomitantly and then continued to multiply following grinding. The number of these two species remained very high until 23 hours while in the later stages Pediococcus cerevisiae was also found. During this stage idli is being steamed and consumed. Thus, these two species are responsible for the acid production and leavening of the batter. The usual aerobic contaminants present on the ingredients are eliminated partly by careful washing and partly by acidic conditions produced by the fermentation. The fermenting microorganisms appear to be present on the ingredients and if the product has to be made daily, there might be some advantage in adding a bit of the freshly fermented batter to the newly ground batter. Yeasts like Torulopsis and Trichosporon can possibly leaven the batter if present in sufficient number, but it is highly unlikely that they produce the acid characteristics of idli.

Jalebi

To make jalebi, refined wheat flour (Maida), dahi and water are mixed into a thick batter and fermented for 14-16 hours. The fermented batter is deep fat fried in spiral shapes and immediately immersed in sugar syrup (600B to 750B) for a minute or two and eaten.

Microbiology of jalebi fermentation

Inoculum in jalebi ads from the natural microflora includes *Lactobacillus fermentum, Streptococcus lactis, Streptococcus faecalis, Lactobacillus buchneri and Saccharomyces spp*.

Changes during jalebi fermentation

During fermentation, pH decreases from 4.4 to 3.3, volume of the batter increases about 9%; both amino nitrogen and free sugar decrease.

Dosa

It is a thin crisp, fried, pancakes like staple food of southern India and is also gaining much popularity in other parts of the country. Like most of the fermented foods consumed in India and other Asiatic countries, dosa is prepared by natural fermentation. Dosa batter is very similar to idli batter, except that both the rice and black gram dhal are finely ground. The batter of dosa is thinner than the idli batter. Following fermentation, the dosa is quickly fried as a thin, fairly crisp pancake and eaten directly. The unflavored crisp pancake may be rolled onto a cooked mixed vegetable mixture and eaten with samber, which is a thin richly spiced soup of dhal and vegetables. It is an important source of protein and calories in the diet and nutrition of south Indians. Since is easily digested therefore it is often used as food for infants and invalids.

Method of dosa preparation

Ingredients: The ingredients used for dosa preparation are similar to as that of idli preparation i.e.rice, black gram dhal, salt and water. Rice may be substituted by wheat, bajra (*Pennisetum typhoideum*), maize, or kodri and black gram dhal may be substituted by sprouted peas, cowpeas (Vigna catjang), field beans (*Dolichos lablab*) or soybeans. Fresh groundnut oilcakes may also be substituted for black gram dhal.

Soaking and batter formation: Generally, equal quantities of rice and dehulled black gram dhal are soaked in water at room temperature separately for 5-10 hours. It is common practice that finely ground powders are used to prepare batter. The finely ground powders are mixed with water at temperatures ranging from 480 to 980 °C, best at 80 °C for the preparation of batter. Water is added in the range of 2.0 to 2.2 times the initial dry weight of ingredients to prepare a batter of viscosity desired for dosa. Salt is added from 0.8 to 1.0% as a seasoning before fermentation.

Fermentation Time: Traditionally, dosa batter is kept overnight for fermentation. The fermentation time should be sufficient to allow a definite leavening and acidification of the batter and to allow for the development of a pleasant acid flavor.

Inoculum: The natural microflora developed during the soaking operation and then at the overnight fermentation is sufficient to leave the dosa batter. However, fermented batter of the previous batch may also be used as a starter culture for fermentation. The microorganisms that develop during the overnight soaking are*: Leuconostoc mesenteroides, Streptococcus faecalis, lactobacillus fermentum*, *Pediococcus cerevisiae, Trichosporon pollutants, Trichosporon beigelii, and Candida kefyr* etc.

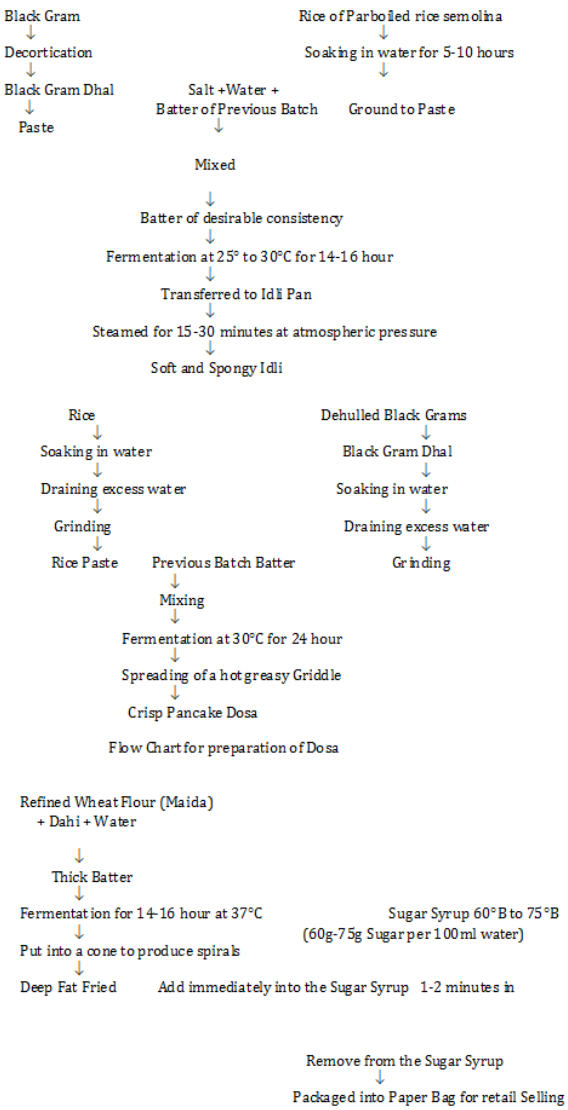
Incubation temperature: Ordinarily, the dosa fermentation is carried out at room temperature. In the tropics, this generally means a temperature of 25-30 °C.

Fermentation containers: In Indian homes, fermentation is customarily carried out in utensils that have sufficient capacity to hold the batter and clean to avoid excessive contamination. The containers are covered with a clean damp cloth to prevent the entry of insects.

Grinding of the Ingredients: Stone mortars are used for grinding the ingredients in Indian homes. These provide excellent control of the particle size in batter. The ingredients are very finely ground to a thin paste and then salt is added in it.

Harvesting and preservation: As soon as the batter becomes leavened and acidified, it is spread onto a hot and greasy griddle where it assumes the shape of a crisp pancake. The spreading of dosa on the griddle is an art that matures with practice. Sometimes a mixture of cooked different vegetable is poured onto the crisp dosa and the sides are rolled, which now becomes ready to be eaten with a spiced soup of dhal and vegetables popularly known as Samber.

Microbiology of dosa fermentation: Traditional dosa batter fermentation has revealed the Occurrence and role of several bacteria alone or in combination with yeasts in bringing about various biochemical changes but Leuconostoc mesenteroides appears in the fermentation early and brings about the leavening in batter. It is also along with Streptococcus faecalis involved in the acid production. These organisms appear to be present on the ingredients and develop during fermentation. Therefore it becomes an advantage that previous batch inoculum is used as a starter for fermentation. Yeasts, if are present in sufficient number can leave the batter but they have no role to play in acid production, which is characteristic of dosa batter fermentation. Pediococcus cerevisiae appear very late in the fermentation when the batter becomes ready to be fried on hot and greasy griddle. **(Flow Chart 9)**



Flow Chart 9

Fermented sausages

Sausages are cylindrically shaped, fermented, solidified, and heat stabilized emulsions formed by mixing together proteins, fats, water, salt and flavorings. Color, flavor and texture of sausages depend on the type of meat, which is mixed and comminuted together with ice, salt, spice flavorings, curing agents and selected meat trimmings, to form a sausage emulsion. The word “sausage” is derived from the Latin, salus; meaning salted or literally, preserved meat. The term “salus” undoubtedly was used by the Romans to denote meat preserved through the use of salt. Basically all sausages are comminuted meats. Products differ primarily because they are spiced in varied fashion and because of differences in methods of processing.

Classification of sausages

Sausages may be loosely classified into three general categories: 1) Fresh sausages. 2) Cooked or Smoked sausages and. 3) Dry Sausages. Some sausages, especially the dry and semi-dry types depend on bacterial fermentation of the production of their characteristic flavors, while in manufacture of some of the more common sausages such as frankfurters bacteria play no role. No distinct classification can be made based on spice formulae because basic spices are used in virtually all products.

Fresh sausages: Fresh sausages are always kept under refrigeration and are fried, boiled, or Cooke thoroughly before serving. They are prepared from selected cuts of fresh meats, principally pork, or beef that has not been previously cured. Examples of fresh sausages are fresh Pork sausages that are prepared selectively from pork meat and fresh sausages that may also contain a percentage of beef and other meats in addition to pork meat. Pork trimmings are put through a grinder, seasoning is added and mixed, and the product is stuffed into natural casings or sold in bulk.

Cooked or smoked sausages: These can be divided into two categories -1) smoked and un cooked and 2) smoked and cooked. Both these categories of sausages are prepared from cured meats and must be kept under refrigeration prior to preparation for serving. Smoked and uncooked sausages -include Smoked country-style Pork sausages that are subjected to mild curing and then placed in casings and smoked and cooked before serving. Other varieties like mettwurst, smoked country-style sausage and polish sausage, all contain beef and pork mixed in various proportions. Smoked and cooked sausages -these are prepared from both uncured and cured meats. They are processed by cooking and smoked after cooking. Examples of these are Frankfurters (60% beef and 40% pork), Bolonga (cured beef and pork), Berliner sausages (cured, coarsely ground pork and finely chopped beef), and German type Mortadella (Cubes of fat pork and pistachio nuts), Liver sausages (pork liver and gelatin), Blood sausages or Bluwurst diced, cooked fat pork plus finely ground cooked meat, blood and gelatin.

Dry sausages: Dry sausages are prepared by curing freshly comminuted meats added with Curing agents and spices for 2 to 3 days. They are then placed in casings and are processed by carefully controlled air-drying. When pork is used in such sausages, it is subjected to a light smoke in order to destroy live trichinae. The principal dry sausages are either salamis or cervelats. Salamis are generally more highly seasoned than cervelats.

Meat Ingredients for sausages: The meat ingredients used chiefly in preparing sausages are those parts of the animal that do not have a ready sale as such. These are classified according to their "water binding" properties i.e. their ability to retain moisture during thermal processing. Meats considered to have best binding properties are skeletal tissue from the beef animal, and include bull meat, shank meat, chucks and boneless cow meat. Medium binders are head meat, cheek meat, and lean pork trimmings. The meat with low binding properties usually contains large proportions of fat or are non-skeletal or smooth muscles. Examples are regular pork trimmings, beef brisklets, heart, giblets and tongue trimmings. Meat tissues classified as "Filler Meats" and considered to have little or no binding properties include ox lips and tripe, pork stomachs, skin, snout, lips, and particularly defatted pork tissue; their use in sausages must be severely limited if the quality of the product is to be maintained. The muscles of beef in sausages enhances flavor as well as contributes to color and texture. The beef muscles contain water-soluble nitrogenous extractives that act favorably on flavor. Fat in the sausages enhances flavor and changes the texture of the sausages. They become tender and juicy, but total fat in sausage must not exceed 50%.

Ice and salt: The addition of ice (moisture) assists in controlling the temperature of the emulsion while it is comminuted. Otherwise the chopping temperature will exceed 60oF, which will lead to instability of emulsion and promote bacterial growth. The moisture in the final cooked sausage should not exceed four times the meat proteins plus 10%. For fresh sausages that have not been heat-processed the limit is four times plus 3%. Water and salt make meat proteins soluble, which then stabilizes the fat globules in the sausage emulsion. Salt also adds to flavor.

Binders or fillers: Cereal flour, potato flour, soy flour, bread or cracker crumbs, milk powder, casein are some of the binders that are used both to hold together and to extend the meat ingredients in the sausages. The flours that are made from cereals as corn, durum wheat, and rye and from potato starch absorb water highly; however, they must not readily ferment when mixed with it. The soy flour with its low fat content enables making sausage flour high in protein content. Rice and cracker flours are also high in protein content. Binders or fillers improve color, provide better binding properties, improve slicing characteristics, change or improve flavor and reduce cost of the product. Their value depends on their ability to absorb moisture in the emulsion and retain it throughout heat processing. The proportion of fillers in sausages should not exceed 3.5%. When corn syrup and milk solids are used their proportion should not exceed 2%.

Curing agents: The common curing agents for sausage preparation are salt, sodium nitrite and/or nitrate and sugar. Three percent of salt on the basis of the meat ingredients is frequently used in sausages. Sodium nitrite and/or nitrate are usually added along with the salt to the beef portion of the sausage emulsion. The proportion of sodium nitrite may not exceed ¼ oz per 100 lb of meat; if sodium nitrate is used with nitrite, not more than 2 oz should be employed per 100 lb of meat. Sugar is added in many sausage products at a level of 0.5-1.0%. Other curing agents such as potassium nitrate (saltpeter), potassium nitrite, vinegar and flavorings are also added.

Seasonings: Variation in seasoning is one factor that is responsible for the large number of sausage varieties. These seasonings may be added as ground natural spices, extracted oils, and oleoresins, or a mixture of the two. Spices that are usually employed include allspice, black pepper, cardamom, cinnamon, coriander, garlic, mace, nutmeg, paprika and sage. Most seasonings for pork sausage contain 1.75-2.0 lb of salt, 6-8 oz dextrose, 4-5 oz pepper, 2-3 oz sage and 0.25 oz of ginger, Per 100 lb of meat.

Other additives: The other condiments used in meat products are pistachio nuts, mono sodium glutamate, ascorbates and isoascorbates. Pistachio nuts are used in headcheese, meat loaves and Braunschweiger. Monosodium glutamate is occasionally employed in pork sausage at a level of 0.1% to enhance flavor. Ascorbic acid or d-isoascorbic acid (erythrobic acid) is used at a level of ¾ oz or ⅞ oz of their sodium salts, in each 100 lb of sausage product. These are commonly used in smoked sausage to assure minimum color development and retention. Coloring matter and dyes, which are approved by the regulatory bodies may be mixed with the rendered fat, applied to animal and artificial casings, and applied to such casing enclosing products. The following coloring matter and dyes are acceptable 1) Natural coloring matters -alkanet, annatto, carotene, cochineal, green chlorophyll, saffron and turmeric 2) Coal tar dyes -all food certified dyes.

Sausage casings: Sausage casings may be natural casings prepared from some part of alimentary tract of cattle, sheep or hogs, or they may be cellulosic casings. These latter are frequently used on frankfurters and may be clear or colored.

a. Method of preparation of frankfurter sausage

Frankfurters are the most popular of all the sausage products. More frankfurters are consumed than any other type of smoked and cooked category of sausage; they represent 25% of all sausage sold. The meat formulation of ordinary frankfurters is 40-60% beef and 60-40% pork. Beef content includes Bull meat, boneless chuck, plates, hearts and trimmings while pork ingredients are filler meats such

as tongue, snout, lips and other byproducts in proportion not exceeding 20%; more will result unsatisfactory product. Fillers employed are dry skim milk, corn syrup solids and sometimes cereals. Total filler content should not exceed 3.5%. Meat used for preparation was pre-cured, but at the present time the curing agents are normally added at the time of chopping. Salt sugar and curing salts are added at the level of 3 lb of salt, ½ lb of dextrose, ¼ lb oz of sodium nitrite and 2 oz of sodium nitrate, for each 100 lb of meat. The more common spices and seasonings used are pepper, nutmeg, mace, cinnamon, mustard and garlic. After chopping and mixing, frankfurters are stuffed into casings and linked. They then held at refrigeration or ambient temperatures for varying periods of time prior to heat processing to permit completion of curing process. Ascorbic acid may be used to obviate the need for this holding period. The sausages are then heated and smoked. Immediately after smoking, they are cooked with a spray of hot water at a temperature of 77-82 °C. Simply raising the smoke house temperature finishes some frankfurters; such dry-processed products are usually given a brief hot water shower to plump the sausages and provide better peeling characteristics. The frankfurters are then cooled by cold water showering to an internal temperature slightly above ambient; the remaining heat is usually sufficient to dry the product prior to its placement in the holding cooler at 2-7 °C. The total processing time may be as short as 65 minutes (when the cure proceeds rapidly) or as long as 2.5 hours. Colored cellulosic casings may be used to add color, or dye may be mixed with the hot shower water, which must then be recirculated; occasionally frankfurters are colored by dipping. Certified coal tar colors can also be used. Frankfurters processed in cellulosic casings may have their casings peeled from them after processing to produce the product sold as skinless variety.

b. History of sausage

Sausage, one of the oldest forms of processed food, was developed some thousand years before the birth of Christ. It started by slow stages from simple process of salting and drying meats by the aborigines and was originated in part as a means of preserving meat that could not once be consumed. The American Indians combined chopped, dried meat with dried berries and pressed these ingredients into a cake for use when food was scarce. Similar drying of meat was a common place along the shores of the Mediterranean centuries before the rise of Roman Empire. The ancient Romans were extremely fond of a sausage made of fresh pork and white pine nuts, chopped fine and seasoned with cumin seed, bay leaves and black pepper. Salami is mentioned in Grecian literature of about 5th century B.C. Sausage was made and eaten by Babylonians (1500 B.C.). The fact that a city of Salamis existed on the east coast of Cyprus in Aegean Sea about 449 B.C. provides foundation for a supposition that salami sausage may have originated in this ancient Grecian city. The various types of sausages as we know today were developed in certain European localities because of local climate conditions. European sausage makers developed products that would keep under the climatic conditions of their particular area. Since artificial refrigeration and canning processes were unknown therefore sausage makers of Italy and southern France, developed dry sausage products. German and Hungarians produced definite types of dry sausages.

c. Action of microorganisms in sausages

Paulo and Smith (1977) have shown that Micrococci dominate the surface of stuffed sausage during the early stages of fermentation. These are killed at pH 5.5 and are not found in the sausage after heat treatment of drying. The results corroborated the research carried out by Sison (1967) in Philippines on the native chorizo-type sausages. The major functions of micrococci during the fermentation are the reduction of nitrate to nitrite and the production of catalase. Lactic acid bacteria rarely reduce the nitrates to nitrites. Lactic acid bacteria were also found in the fermentation. The activity of the lactic acid bacteria is the conversion of sugars to lactic acid by EMP pathway. Streptococcus diacetylactis produces diacetyls and acetoin that imparts nutty flavor and aroma to some sausage. *Staphylococci* also actively reduce nitrates to nitrites. Excessive nitrite level in sausage has been noted when using high nitrate concentrations with micrococci, producing a defect in sausage called “nitrite burn”. The micrococci are also lipolytic, and they produce lipase during early stages of fermentation. This results in an increase in free fatty acids, volatile fatty acids and carbonyl compounds after 28 days of drying. Lactic acid bacteria produce varying amounts of hydrogen peroxide, which is destroyed by the catalase of *micrococci*. Another important function of lactic acid bacteria is the inhibition of Staphylococci. This inhibition or suppression also suppresses the enterotoxin production by Staphylococci. This inhibition is more pronounced as the ratio of lactic acid bacteria to Staphylococci increases and as temperature of the fermentation decreases. The beneficial effects of lactic acid bacterial starter cultures in inhibiting Staphylococci and enterotoxin production in fermented sausages have also been demonstrated.

Sauerkraut

These are German terms for sour cabbage, which is generally prepared from shredded cabbage. The yellow -white shreds are approximately 2-5 mm in width and as long as 20 cm.

Standards for sauer kraut

It must contain at least 0.75% lactic acid and less than 10% of the total acid can be volatile. The pH must not exceed 4.1. The strainable brine should amount to about 10% of the total weight of sauerkraut and should contain from 0.7 to 3.0% NaCl.

Preparation for fermentation

Properly matured sound heads of cabbage are trimmed to remove the damaged parts and outer green or dirty

leaves. The cabbage is then sliced to shreds of size 0.16 to 0.08 cm in thickness. The shredded cabbage is conveyed to vats or tanks for salting and fermentation.

Role of salt

Salt plays a primary role in the preparation of sauerkraut; therefore its concentration is carefully controlled. According to legal standards salt must not be less than 2% and must not be more than 3%. Most of the producers of sauerkraut add salt in the concentration of 2.25 to 2.5%. Salt extracts water from shredded cabbage by the process of osmosis, thus forming fermentation brine. It suppresses the growth of some undesirable bacteria that might cause deterioration of the product and at the same time makes conditions favorable for the growth of lactic acid bacteria. Salt also contributes to the flavor of finished product by yielding a proper salt acid ratio. The use of too little salt causes softening of tissues and produces a product lacking in flavor. Too much fermentation and over salting may produce a product with a sharp, bitter taste. It may also cause darkening of the color of product and may favor the growth of pink yeasts. Brine begins to form once the shreds are salted and tank is closed when it is filled to the proper level. Then a weight is placed over these shreds so that it squeezes the water out of the shreds. The weight may be of wood (old method) or some plastic bag filled with water may be placed (modern method).

Microbiology of sauerkraut fermentation

Pederson first described the lactic acid bacteria that he observed in fermenting sauerkraut. He found that the fermentation was initiated by the species of Leuconostoc mesenteroides. This species was followed by gas forming rods and finally by non-gas forming rods and cocci. Leuconostoc mesenteroides is a heterofermentative bacteria and it grows more rapidly than other lactic acid bacteria. It is active over a wide range of temperature and salt concentrations. It produces acid and carbon dioxide that rapidly lowers the pH, thus inhibiting the activity of undesirable microorganisms and enzymes that may soften the shredded cabbage. The carbon dioxide replaces the air and creates an anaerobic condition favorable to prevent oxidation of ascorbic acid and natural color of the cabbage. It also stimulates the growth of many lactic acid bacteria. While this initial fermentation is developing, the heterofermentative species of lactobacillus brevis and homofermentative species of lactobacillus plantarum and sometimes Pediococcus cerevisiae begin to grow rapidly and contribute to the major end products like lactic acid, carbon dioxide, ethanol, acetic acid. Minor products also appear in the fermentation. The minor products are a variety of volatile compounds e.g. diacetyls, acetaldehyde and primary carbonyls.

Control of fermentation

Temperature, salt concentration and sanitary conditions are the primary environmental factors controlling the sauerkraut fermentation.

Influence of Temperature

At low temperature (7.5 °C), fermentation is very slow. Leuconostoc mesenteroides grows slowly attaining an acidity of 0.8-0.9% in terms of lactic acid in a month. Acidity is important for its preservative effect. Other lactobacilli and pediococci cannot grow at this low temperature. The sauerkraut may not be completely fermented for 6 months or more or until the temperature rises to a temperature suitable for the growth of higher lactic acid producing lactics. At a temperature of 18 °C with a salt concentration of 2.25%, a total acidity of 1.7-2.3% as lactic acid will be attained, with an acetic to lactic acid ratio of about 1:4 in about 20 days. At higher temperature i.e. 23 °C, the rate of fermentation will be greater so that a brine acidity of 1.0-1.5% (lactic acid) may be attained in 8 to 10 days. Active growth of Lactobacillus plantarum and Lactobacillus brevis may be initiated in 3-5 days and the kraut may be completely fermented in approximately one month. At still higher temperature of 32 °C, the rate of fermentation may be very rapid and an acidity of 1.8-2.0 may be attained in 8 to 10 days. The major share of the acid produced will result from the growth of homofermentative bacteria *L. plantarum* and *P. cerevisiae*. The flavor of the sauerkraut will be inferior, similar to an acidified cabbage. At the higher temperature sauerkraut will darken rapidly unless canned immediately. It will have a poorer shelf life than sauerkraut fermented at lower temperature. This sauerkraut also has low percentage of acetic acid and will not attain as high a total acidity, even though the pH is lower. It will also subject to yeast spoilage, partly because of its low content of carbon dioxide. It is also low in ascorbic acid content.

Influence of salt

Salt plays a primary role in the preparation of sauerkraut; therefore its concentration is carefully controlled. According to legal standards salt must not be less than 2% and must not be more than 3%. Most of the producers of sauerkraut add salt in the concentration of 2.25 to 2.5%. Salt extracts water from shredded cabbage by the process of osmosis, thus forming fermentation brine. It suppresses the growth of some undesirable bacteria that might cause deterioration of the product and at the same time makes conditions favorable for the growth of lactic acid bacteria. Salt also contributes to the flavor of finished product by yielding a proper salt acid ratio. The use of too little salt causes softening of tissues and produces a product lacking in flavor. Too much fermentation and over salting may produce a product with a sharp, bitter taste. It may also cause darkening of the color of product and may favor the growth of pink yeasts. Brine begins to form once the shreds are salted and tank is closed when it is filled to the proper level. Then a weight is placed over these shreds so that it squeezes the water out of the shreds. The weight may be of wood (old method) or some plastic bag filled with water may be placed (modern method).

Acetylcholine content in sauerkraut

Sauerkraut is known to provide certain laxative properties; both sauerkraut and its juice have been used as purgative. The strain of l. plantarum produces acetylcholine in the presence of choline, while simultaneously fermenting carbohydrates. This acetylcholine is of significance in nerve activity.

Defects and spoilage of sauerkraut

Pink-Kraut: Pink-kraut was observed first by Butjagin (1904) Wchmer (1905) and Henneberg

1916. Brunkow et. al (1925) and Fred and Peterson 1922 noted that this cause was the growth of pigmented yeast i.e. asporogenous yeasts presumably members of genus Rodotorula. Pederson and Kelly (1938) observed that Pink-kraut usually contained a salt content greater than 2.5%. They associated the growth of yeasts with any factor that would inhibit a normal fermentation or that would suppress or adversely affect the heterofermenting bacteria. Sometimes pink-kraut was observed in vats of sauerkraut only a few feet away from an area of soft-kraut. The latter condition arises due to insufficient salt concentration. Stamer (1975) reported that L. brevis produces a red pigment under certain condition that can be related to discoloration or darkening of sauerkraut. The red color occurs between pH 4.4 and 5.2 and is most readily generated under aerobic conditions. Chemical reducing agents like ascorbic acid, cystein or glutathione inhibit this color formation.

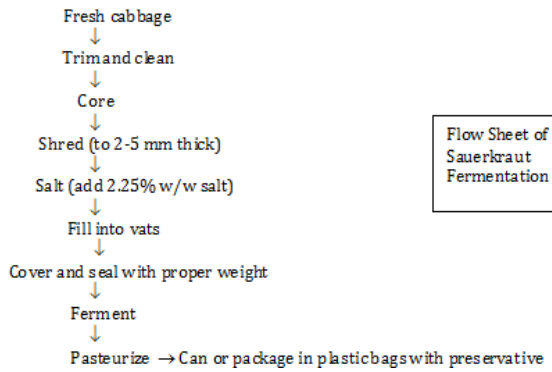
Slimy or Ropy Kraut: This is generally caused by dextran formation induced by the *L.mesenteroide* and is transitory in nature. This species prefers to ferment fructose rather than glucose, therefore in the fermentation of sucrose; the fructose is fermented leaving the glucose which interacts to form slimy, ropy water insoluble dextrans. These vary from an almost solid, gelatinous mass to ropy slime surrounding the bacterial cell.

Other Defects: Discoloration caused by autochemical oxidation. Loss of acidity caused bgrowth of molds and yeasts. Spoilage caused by molds and yeasts cause off-flavors and off-odors (yeasty and rancid). Slimy, softened kraut caused by aerobic growth of asporogenous yeasts.

Advantages of the acid food fermentations: 1) They render foods resistant to microbial spoilage and development of food toxins 2) They generally preserve the food between the time of harvest and consumption 3) they make the food less likely to transfer pathogenic microorganisms 4) they modify the flavor of the original ingredients and often improve the nutritional value. **(Flow Chart 10)**

Kimchi

Kimchi is the general name given to a group of fermented acid vegetable foods with a long tradition in Korea. More specific names are used for these pickled vegetables depending on the raw materials, processing methods, seasons and localities. Most kimchi is prepared at household level and consumed directly while limited amounts of cabbage-based kimchi are canned in factories and sold in the market. Kimchi is available throughout the year and, served three times a day, is a staple in the diet along with cooked rice and accessory side dishes. It is a favorite food unique in its complex of sour, sweet and hot pepper flavors accompanied by carbonation derived from fermentation with natural microflora. Kimchi differs from sauerkraut in two respects: 1) It has, optimally much less acid and 2) It is carbonated.



Flow Chart 10

Methods of preparation for typical korean kimchi (Tongbaechu-Kimchi or Kakduggi-kimchi)

Materials for kimchi preparation include 1) fresh vegetables (major vegetables are Korean cabbage, and radish; minor vegetables are garlic, green onion, ginger, leaf mustard, hot pepper, parsley pear, chest nut and carrot), 2) Jeotkal (Korean pickled fish) 3) fresh fish 4) seasoning agents (table salt, sesame seeds, sugar, monosodium glutamate, chenggak (type of seaweed), pear etc. additional minor ingredients may also be added depending on the household maker these are: saeujeot (pickled shrimp), meolchijeot (pickled anchovy), whangsegijeot, frozen Pollack, oyster, shrimp and small octopus. The ratio of major to minor ingredients varies depending upon the household maker; the range generally is 70-90 to 30-10. Although the proper combination of minor ingredients is reported to be the key to good-tasting kimchi, the most important factor seems to be the salt concentration. Salting of cabbage can be done at 5 to 7% for 12 hours or in 15% saline solution for 3-7 hours followed by rinsing and draining. Optimum salt concentration during kimchi fermentation is approximately 3% and is adjusted by experience at the household level. Fermentation of kimchi in the homes is usually carried out at ambient temperature. Using 3% salt concentration, the optimum fermentation period is one day at 30 °C, 2 to 3 days at 20 °C, 12-15 days at 10oC and 30-60 days at 5 °C. Optimum acidity of kimchi is 0.4 to 0.8% (as lactic acid). Higher acidity makes the product unacceptable.

Microorganisms

Kimchi is fermented by the microflora of the region. Organisms isolated include lactic acid bacteria, such as

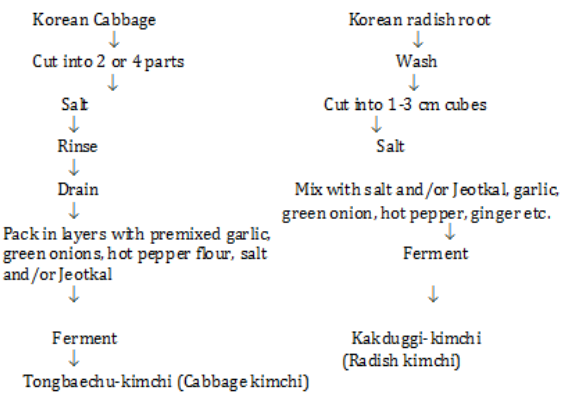
Leuconostoc mesenteroides, Streptococcus faecalis, Lactobacillus brevis, Pediococcus cerevisiae, *Lactobacillus plantarum*, and aerobic bacteria, such as *Achromobacter, flavobacterium and pseudomonas spp*. In the later stages of fermentation yeasts and molds appear that are reportedly causes of softening.

Preservation of kimchi

Kimchi is preserved at low temperature (below 5 °C) for a very short period of time. During storage at elevated temperature, rancidity and soft rot are accelerated by the microbial action. Thus shelf life is very short in summer months.

Biochemical changes in kimchi

The initial pH 5.5-5.8 falls to an optimum of 4.5-4.0. Optimum acidity (as lactic acid) is 0.4-0.8%. Salt concentration remains constant during fermentation. Kimchi fermented at low temperature (6-7 °C) contains more lactic, succinic, oxalic, tartaric, malonic, maleic, and glycolic acid than that fermented at 22-23 °C. Vitamins B_1, B_2, B_{12} and niacin reach the highest levels (twice the initial level) when kimchi possesses the most palatable taste and decrease when kimchi becomes sour. Vitamin C and carotene content decreases upon ripening. **(Flow Chart 11)**



Flow sheet of Tongbaechu-kimchi and Kakduggi-Kimchi

Flow Chart 11

Cucumber Pickles

The cucumber (Cucumis sativus) is popular both as a fresh and as a pickled vegetable. It is grown widely in temperate climates although originally of semitropical origin. Cucumbers for pickling must be grown from varieties known to have regular form, firm texture, and good pickling characteristics. Earlier varieties used for pickling were monoecious plants but new varieties developed by hybridization method have preponderance of female flowers and are called Gynoecious. These new cultivars often have greater vigor and uniformity than the open pollinated ones formerly grown. In addition, several of the hybrids are early maturing so they can be used to advantage in harvest scheduling. Pickling cucumbers are harvested while still immature. Fully grown (ripe) ones are undesirable for pickling because they are too large, change color and shape, are full of mature seeds, and are too soft for most commercial uses. After harvesting the cucumbers are immediately transferred to the salting station to avoid sweating i.e. growth of undesirable softening microorganisms. Unsound, decomposed, broken or crushed, distorted (wilt, rots, crooks, nubbins, etc.) are sorted out. They are then graded in a mechanical grader into 4 or more sizes. Three types of cucumber pickles are made: 1) Fresh pack -These are held in salt brine for as long as 2 days, then packed into jars or cans and pasteurized. They undergo marginal fermentation. 2) Salt Stock Pickles -From these pickles a variety of processed products are prepared. They undergo complete lactic acid fermentation. 3) Fermented Dill Pickles -They also undergo complete lactic acid fermentation. Dill herb is also added in these types of pickles.

Brining techniques for salt stock

There are two general methods for preparing salt stock pickles for fermentation these are Dry Salting and Brining

Dry Salting

For cucumbers, dry salting is done after first adding salt brine to cover the bottom of the tank (at least 12 in.) to form a cushion. This prevents bruising, breaking, or crushing the fresh cucumbers. Dry salt is then added at the rate of about 22.5 kg for every 450 kg of small cucumbers and 29.25 kg for every 450 kg large cucumbers. When full, the tank is covered with a wooden lid very tightly. Brine forms by osmosis. If the brine formed by osmosis does not cover cucumbers then 400 salometer brine is added to the desired level. The brine should be recirculated a day or two after tank is filled in order to equalize the concentration of salt throughout the brine. For long storage 60o salometer brine is used. In industry 100 salometer is equal to 2.64% NaCl by weight or 100o salometer is equal to 26.359 g NaCl at 15.5°C (saturated solution of salt).

Brine salting: Brine salting process is preferred over dry salting because dry salting yields soft,

flabby, shriveled pickles that do not fill out properly when processed. Therefore most picklers mostly use brine-salting technique for fermenting cucumbers. For brine salting 'low' or 'high' brine process may be used. In low brine salting, a salt brine of 25-30°C salometer is added into cucumbers whereas in high brine technique, 40°C salometer salt is added into cucumbers and tank is closed to air tight first by covering it with polythene sheet and then by lid. The cucumbers are handled by the same procedure as described in dry salting except brine is used to cover the cucumbers. Now a days, molded plastic and fiberglass tanks are being used in place of wood or concrete tanks. These plastic and fiberglass tanks have several advantages. These are 1) They are not subject to biological degradation or metal corrosion 2) They do not have to be maintained during the

off season, as do wooden tanks 3) As all the valves and piping are made of plastic, the problem of metal corrosion and contamination is eliminated 4) They are properly designed, closures are nearly airtight so problems of loss of acidity is reduced.

Microbiology of the cucumber fermentation

A rapid development of the microorganism causes a spontaneous fermentation as soon as the lid of the tank is closed after adding brine. The rapidity of fermentation is directly related to the temperature of the brine, concentration of the salt in brine, availability of fermenting materials and relative number of microorganisms available on cucumbers. Fresh cucumbers contain numerous and varied microflora including many potential spoilage microorganisms and a small number of lactic acid bacteria (5-103 acid forming bacteria per gram of cucumber). When cucumbers are brined at 5-8% NaCl range and allowed to undergo natural fermentation, the salt solution supports the fermentation by a sequence of various types of microorganisms. This sequence is categorized into four stages 1) Initiation 2) Primary fermentation 3) Secondary fermentation and 4) Post-fermentation

Initiation

This stage may include growth of many facultative and strictly anaerobic microorganisms originally present on the fresh material, the growth of undesirable microorganisms such as Gram-negative and spore-forming bacteria is inhibited as the pH gets lowered and lactic acid bacteria become established. The quality of the final product depends largely of the rapidity with which lactic acid bacteria are established and undesirable bacteria are excluded.

Primary fermentation

In this stage lactic acid bacteria (*Leuconostoc, Lactobacilli and Pediococci*) and both fermentative and oxidizing yeasts are the predominant active microflora. They grow in brine until the fermentable carbohydrates are exhausted or until there is production of lactic and acetic acids. In normal fermentation the undesirable microorganisms are excluded within 10-14 days. Buffering capacity and the fermentable carbohydrate content present in the medium are the important factors that govern the extent of fermentation by lactic acid bacteria and the extent of subsequent fermentation by yeasts.

Secondary fermentation

Pediococci, Lactobacillus brevis, and Lactobacillus plantarum and fermentative yeasts are responsible for the completion of lactic acid build up in this final stage of the fermentation. The acid tolerant yeasts still remain in the medium after the lactic acid bacteria are inhibited by low pH values and continue to grow till fermentable carbohydrates are exhausted.

Post fermentation

When fermentable carbohydrates are exhausted, microbial growth is restricted to the surface of brines exposed to air; the spoilage bacteria may become established on the surface of improperly managed tanks. At the end of fermentation, total acidity is 0.9% and pH is equal to 3.3.

Fermented dill pickles

Cucumbers when are subjected to bacterial fermentation in dill flavored, spiced, salt brine; the product obtained is called Dill pickles. They have their distinctive flavor and aroma due to the products of fermentation of lactic acid bacteria and to the blending of flavor and aroma of dill herb and spices that were added to the brine.

Method of preparation: The larger size cucumbers are washed and placed in suitable containers, together with the requisite amount of dill weed (which was earlier cured in vinegar, salt and brine) and dill spice and brine solution. Dill pickles are generally fermented in low salt (5% NaCl) brine solution. Vinegar is added to retard the growth of undesirable microorganisms (by decreasing the pH value). The fermentation is carried out at a temperature between 21 °C and 26.7 °C for 3-4 weeks. A curing period of 3-4 weeks further is also necessary. During this period, the flesh of pickles becomes entirely translucent and acidity about 0.5-1.2% (lactic acid). In addition, there is small amount of volatile acid (acetic acid); lactic acid bacteria and yeasts produce ethanol and other minor products.

Microbiology

In the beginning, a wide variety of unrelated microorganisms start growing in the fermentation but soon lactic acid bacteria predominate them. At low temperatures Leuconostoc mesenteroides play an important role in the fermentation. Once Leuconostoc starts predominating other species like Lactobacillus brevis, Lactobacillus plantarum begin to grow in the fermentation and ultimately complete the fermentation.

Packaging

Fermented dill pickles are marketed in bulk plastic containers and glass containers covered with acidified brine, closed and pasteurized at 74 °C for 15 minutes.

Spoilage of cucumber pickles

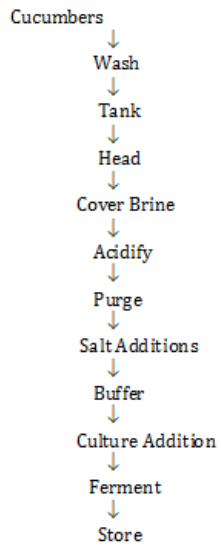
In Cucumber pickles, most of the deterioration is caused by the microorganisms; chemical defects are generally caused by metallic contamination, auto-chemical and physico-chemical reactions. Microorganisms damage the tissues by their cellulolytic or pectinolytic enzymes that result in loss of texture of firmness. Gaseous deterioration caused by microorganisms, resulting in the production of internal cavities or distorted stock caused by excessive gas pressure is another common spoilage. This defect is known as bloater or floater spoilage.

Softening of cucumber pickles

Softening occurs most frequently after brining of the cucumbers for dill or salt stock pickles. The entire skin of the cucumbers become slippery and can be removed easily. Such condition of pickles is sometimes referred to as 'Slip or Slippery pickles' in industry. When softening progresses into the deeper layers of cells and more and more pectic materials, present in the middle lamella separating the individual cells of the cucumber are attacked, the condition is known as 'Mushy Pickles'. A variety of bacteria of Gram-positive type (*Bacillus subtilis, B.pumilis, B.polymyxa, B.stearothermophilus*) and yeasts such as Saccharomyces fragilis, and Rhodotorula that produce pectinolytic and cellulolytic enzymes cause mushy deterioration.

Gaseous Spoilage of Cucumber Pickles

A number of genera of bacteria and yeasts cause gaseous spoilage in cucumber pickles. Undesirable yeasts produce gas because they utilize lactic acid and cause a rise in the pH. The fermenting yeasts have been identified as belonging to genera Brettanomyces, Hansenula, Saccharomyces and Torulopsis. Among bacteria, Lactobacillus brevis, Lactobacillus plantarum cause serious bloater spoilage. The major gases formed during spoilage are generally hydrogen and/or carbon dioxide. Nitrogen purging of the fermenting brine is used to reduce undesirable levels of carbon dioxide that otherwise might result in bloater formation. **(Flow Chart 12)**



Flow Sheet for Brine fermentation of Cucumbers.
Steps that have been added to the overall natural fermentation to render 'controlled fermentation' are indicated in bold face.

Flow Chart 12

Production of Industrial Enzymes

Introduction

Enzymes are biocatalysts produced within the living cells to bring about specific chemical changes. They are present in all living cells and the metabolic reactions common to all cells are catalyzed by these enzymes. The use of enzymes in industry dates back to some centuries before the discovery of enzymes. For example use of barley malt for starch conversion in brewing is a notable example. The field of enzymology was opened by Buchner brothers who showed that cell free extracts from yeasts fermented sugar to produce alcohol and carbon dioxide. In oriental countries microorganisms are directly employed as enzyme source for the preparation of products such as shoyu, miso, natto, and sake. Other important processes in which enzymes are primarily used are cheese making, leavening of bread, manufacture of vinegar, tanning of leather etc. It was Takamine who laid the foundation for the industrial production of microbial enzymes by developing process for producing diastase from fungi. Boidin and Effront of France were the first to produce industrial enzymes from bacteria. So far more than 1300 enzymes have been identified, out of which nearly 100 have been obtained in crystalline form.

Commercial production of industrial enzymes

Selection of microorganisms: The potential microorganisms are isolated from soil, decaying Organic matter or air and are tested individually for their capability to produce the desired product; this process is called primary screening. A few members of this potential natural isolates will possess the desired characteristics. It is also customary to grow the selected organisms on their substrates. In certain cases such as in the case of pectinases the organisms is induced to secrete the desired enzyme. The selected strains are maintained in pure form by lyophilization, on agar slant, or soil culture. The isolates are periodically checked for purity and for the retention of their original activity. Secondary screening is conducted in flasks or small fermentors. This evaluates the true potential of the organism to produce the desired product both qualitatively as well as quantitatively. Once potentiality of the organism is established, investigations are undertaken to work out a suitable medium and optimization of other conditions like pH, temperature, aeration etc. for the maximal enzyme production are carried out. Continuous maintenance of strains may cause degeneration and ability to produce the desired enzyme. Therefore, periodic re-isolation and reevaluation is necessary. The strains are also continually improved to enhance their capabilities by various physical agents such as UV treatment or by chemical methods. Recombinant DNA technology has also playean important role in the strain improvement programme.

Methods of cultivating microorganisms: Several methods of culturing the microorganisms are being employed industry that can be classified as follows: 1) Solid Culture -i) Conventional koji culture ii) Bulk koji culture iii) Rotary drum culture 2) Liquid culture -i) Stationary ii) Submerged These methods are explained in the coming section.

Preparation of starter culture: Working cultures are first prepared from stock culture (i.elyophilized or soil culture). These cultures are tested for their potency from time to time. In certain countries like Japan, there are firms that specialize in supplying pure fungal spores called Tane koji of desired strains of fungi to large manufacturing units. In such cases spores are directly inoculated into the growth medium. Starter inoculums for large fermentations (both for solid and liquid fermentations) have to be progressively built up. The quantity of inoculum required depends upon the batch size and it varies between 0.01 to 0.001 of the volume of the medium and is expressed as number of cells, weight of cell mass or just on the volume basis. Generally the amount of inoculum is kept as low as possible. Large Scale Mold Fermentation using Fungi

Bran process: Wheat bran is moistened with 0.2 to 0.3N HCl and autoclaved at 15lb pressure for1 hour. Addition of acid improves sterilization and inhibits growth of undesirable microorganisms. Sterilization can also be carried out by direct injection technique and continuously stirring the mass SO that bran particles come in direct contact with steam. When dilute acid is used for moistening bran, it is sufficient to hold the medium for 15-30 minutes in live steam to obtain practical sterility. The cooked bran is then cooled to room temperature and inoculated with inoculum grown earlier, at 1% level. It has been reported that 0.4% dry spore inoculum is sufficient for good growth of fungus. The inoculated bran is mixed well and transferred to trays having false bottoms. Layers of 5 centimeter are considered good for uniformly good growth. The trays are placed one above the other 8 to 10 cm apart. Spores germinate within 3-4 hours and temperature begins to rise after 5-6 hour. Aeration is started at this stage and continued till growth is completed. It takes 48-120 hours for the fermentation to complete depending upon the microorganism. After the completion of growth, the trays are shifted to drying tunnels with a central exhaust. The hot air is blown into the tunnel and air temperature is not allowed to rise above 40 °C. Different modification of the above mentioned process are adopted by different enzyme producing companies.

Bulk-koji process: In this method the fungus is cultivated on thick layers of bran up to 25cm t50cm high. The chamber has a false bottom and air is circulated under pressure from the bottom of the chamber. The chambers have a floor area of 2 to 30 meters by 6 to 10 meters. Temperature and humidity of the air in the chamber are automatically controlled.

Rotary drum method: In this method, the fermentation vessel consists of a rotating drum fitted with baffles so that the bran can be stirred. The chamber is also provided with cooling coils and an inlet for aeration. Bran is loaded into the drum and moistened with dilute acid and sterilized with steam. After cooling, the inoculum prepared in bran culture is added and mixed. After charging, the air is passed slowly into the drum which is maintained at a temperature of 28-30 °C. The spores germinate within 5-6 hours during which period drum is rotated slowly for 15-20 minutes after every 2 hours. The drum is rotated continuously for 5-6 hours at a speed of 1 rpm or less. The growth of the fungus is completed within 60 hours. The moldy bran is then spread in the form of layers on paper for drying. After drying, the moldy bran is ground and utilized as such, as an enzyme source or to extract the enzyme.

Extraction process: Powdered moldy bran prepared from the foregoing procedures is utilized for extraction and purification of the enzyme. The moldy mass is extracted with cold water (1-2 °C) or with solvents like ethanol. The common procedure is to employ counter current extraction system which gives a better and clear extract of enzyme. The quantity of water utilized generally is 5-10 times the weight of the moldy bran. The clear extract thus obtained is utilized for concentration and purification of the enzyme.

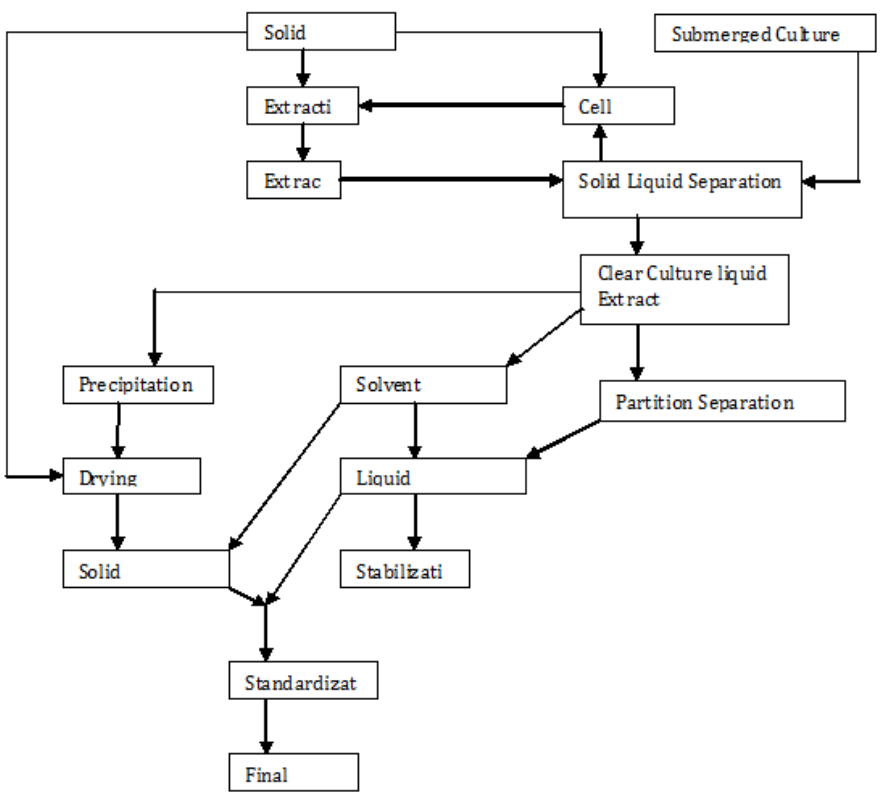
Submerged fermentation: After selecting a suitable microorganism capable of producing the desired enzyme and standardizing the conditions for its maximum output, large scale fermentation is taken up. This involves various operations that are discussed below: The substrate for growing microorganism is designed, based on the availability of cheap raw materials and types of enzyme to be produced. Composition of media recommended for different enzymes consists of components selected from starch hydrolysates, wheat bran extract, milled cereal products, soybean meal, peanut meal, corn steep liquor, distiller's solubles, yeast extracts and other organic and inorganic nitrogenous compounds and mineral salts. The liquid nutrient medium is charged into cleaned fermentors and sterilized by means of steam. The medium is constantly kept stirred during sterilization. The sterilization is carried out for 2-3 hours depending on the size of the batch. Generally the holding temperature is 121 °C for 20 minutes. For large fermentors a continuous high temperature and short time regime is adopted. Direct heating with steam is preferred when medium is thick and viscous. After sterilization medium is cooled to the desired temperature and pH is adjusted to optimum, the inoculum is introduced under aseptic conditions. In commercial production of microbial enzymes generally aerobic microorganisms are used for fermentation. In such cases aeration and agitation is started soon after inoculation. The fermentor is kept under constant pressure to avoid contamination. The agitator speed and quantity of air depends on the size of the batch, medium composition, and the requirements of the selected microorganism. After inoculation microorganisms establish themselves and passing through a lag phase, begin to grow exponentially. The standard fermentors are provided with aseptic sampling devices that allow samples

to be withdrawn for routine checking of contaminants, cell population, morphological observations and enzyme yield. Fermentation is carried out for 1-7 days. Most of the enzymes are secreted into the medium by the microorganism and extracellular enzymes appear in the medium during early part of the logarithmic phase. In case of intracellular enzymes cell disruption techniques are employed to recover the enzymes. The enzyme rich material obtained in any of the three procedures i.e. moldy bran, cultural extract, or cell autolysates is concentration and purified after solution is stabilized by the addition of chemical s such as ammonium phosphate, ascorbic acid, calcium salts, hydrochloric acid, phosphoric acid, sodium citrate, sodium phosphate, sodium sulphite or organic substances such as gelatin, gums, Arabic gum etc.

Purification of enzymes: The fermentation solution is subjected to centrifugation or filtration to obtain a clear liquid free of suspended particles. Concentration of the enzyme is brought by employing techniques such as vacuum evaporation and fractionation. In fractionation, all substances in the crude enzyme solution except the desired enzyme are separated out. Enzyme is then purified by precipitation, absorption, and crystallization. Precipitation of enzyme is carried out with salts such as ammonium sulphite, magnesium sulphite, sodium sulphite, or by other substances added to bring the pH of the solution to its isoelectric point to separate the protein. The former process is called as 'Salting Out' and latter referred to as 'Isoelectric Precipitation'. Water soluble solvents such as ethanol, methanol, isopropyl alcohol, acetone, dioxane etc. are also used for precipitating the enzyme. The precipitated enzyme is separated by process of centrifugation or filtration. The organic solvents are removed by low temperature drying and the inorganic salts by dialysis. Enzyme now gets concentrated manifolds. To obtain an enzyme in pure form, the enzyme solution is absorbed on ion exchange resins, inert earth, calcium phosphate, aluminum hydroxide, colloidal iron etc. after adjusting the pH to the optimum level. Highly purified enzyme is freeze-dried or crystallized.

Enzyme activity

Commercial enzymes are evaluated according to their specific activity per unit volume or weight. The activity is usually measured in 'Enzyme Units'. An enzyme unit indicates an amount of chemical change catalyzed by a definite quantity of enzyme. The activity can be standardized by blending the enzymes with inert materials such as diatomous earth, glucose, sucrose etc **(Flow Chart 13)**.



Flow Chart 13

Production of Vitamins

Riboflavin

Microbiologically produced Riboflavin has long been available in yeast and related preparations in association with many other vitamins of the B-complex category. Riboflavin is essential for the growth and reproduction of both humans and animals. Thus it is often incorporated into the feed of the animals. By fermentation process the riboflavin content of the medium can be raised up to 7 gm/L. Various microorganisms involved in the fermentation of Riboflavin are as follows: a) Ascomycetes- Ermothecium ashbyii and Ashbya gossypii are the commercial strains. b) Bacteria- recovered from the acetone butanol fermentation e.g. *Clostridium butylicum, C. acetobutylicum* and Mycobacterium smegmatis also produce riboflavin. c) Yeasts- *Candida guilliermondia, C. flareris* and Mycocandida riboflava are the non-commercial strains. (Figure 3)

Fermentation process for ascomycetes

Ascomycetes for the production of Riboflavin require semi purified sugars (glucose) and crude organic nutrients like corn steep liquor, animal stick liquor and meat scraps. Glucose may be totally replaced by corn oil, however low levels of corn oil may be added to the glucose to stimulate riboflavin yields. pH is adjusted to 6.5-7.5 and temperature 26-28 °C for 4-5 days. The fermentation is submerged, aerated but high levels of aeration may inhibit mycelial production and reduces the product yield. When Candida spp. are used for riboflavin production, the vessels made of steel cannot be used because the organism is very sensitive to traces of iron, therefore the vessels may be lined with plastic. Cobalt at proper concentration (stimulates the ascomycete fermentation) can be added into the fermentation broth as it partially counteracts the iron toxicity. Candida fermentation can be carried out at low pH, which eliminates the bacterial contamination and less sterilization is required.

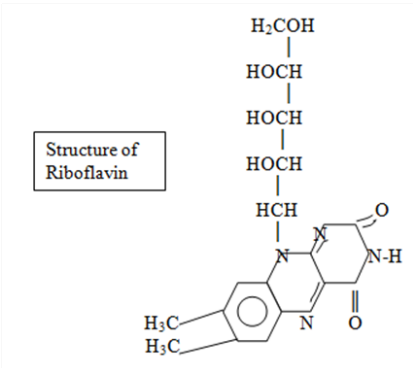
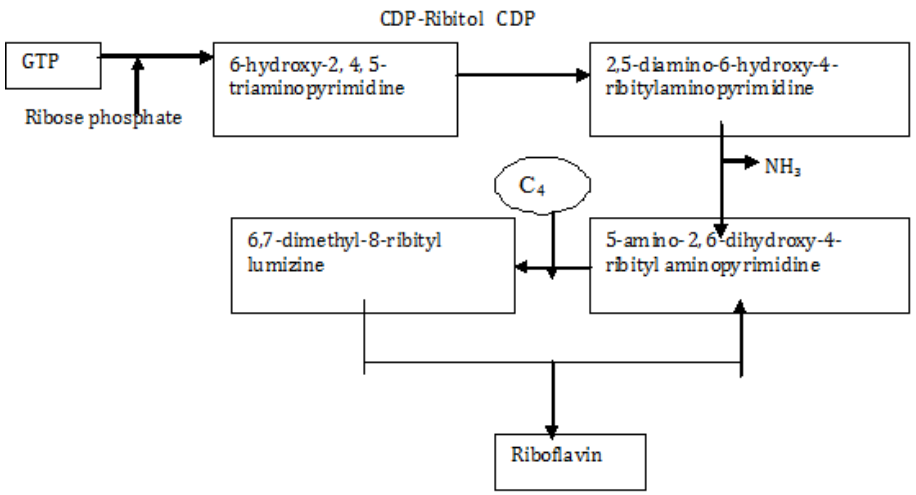


Figure 3

Mechanism of riboflavin accumulation

Kaprálek (1962) and Stárka (1957) demonstrated that fermentation of riboflavin progresses through three phases. First phase is a rapid growing phase with no riboflavin production. The substrate is utilized and oxidized; pH decreases as pyruvic acid accumulates. In the second phase glucose gets exhausted, sporulation begins, pyruvate decreases, ammonia accumulates because of deaminase activity, pH becomes alkaline, Rapid synthesis of cell bound riboflavin occurs in the form of FAD and rapid catalase activity causes disappearance of cytochromes. In the third phase autolysis of the cells occurs with the release of riboflavin into the medium **(Flow Chart 14)**.



Mechanism of Riboflavin Accumulation

Flow Chart 14

Recovery of riboflavin

On completion of fermentation, the solids were dried to a crude product for feed supplement. For a crystalline product, broth is heated for 1 hour at 15lb pressure to solubilize the riboflavin. Insoluble matter was removed by centrifugation and riboflavin is recovered by conversion to the less soluble form by chemical and microbiological methods. The precipitated riboflavin was then dissolved in water or polar solvents or in an alkaline solution, oxidized by aeration and recovered by recrystallization from aqueous or polar solvent solution or by acidification of the alkaline solution.

Carotenoid

Carotenes

Carotenes are precursors of vitamin A. Some carotenes are normally present in foods and have an essential biological function to perform. They are used as food supplement to prevent or cure vitamin deficiency diseases. In addition other pigmented carotenoids are used both as food additives for intensifying or modifying the color in fats, oils, cheese, and beverages and also as animal feed supplement to enhance the color of such foods as egg yolks and chicken flesh. Though carotenoids are widely found in plants and animals, only microorganisms and plants have the necessary systems to synthesize a wide range of these products.

Microorganisms

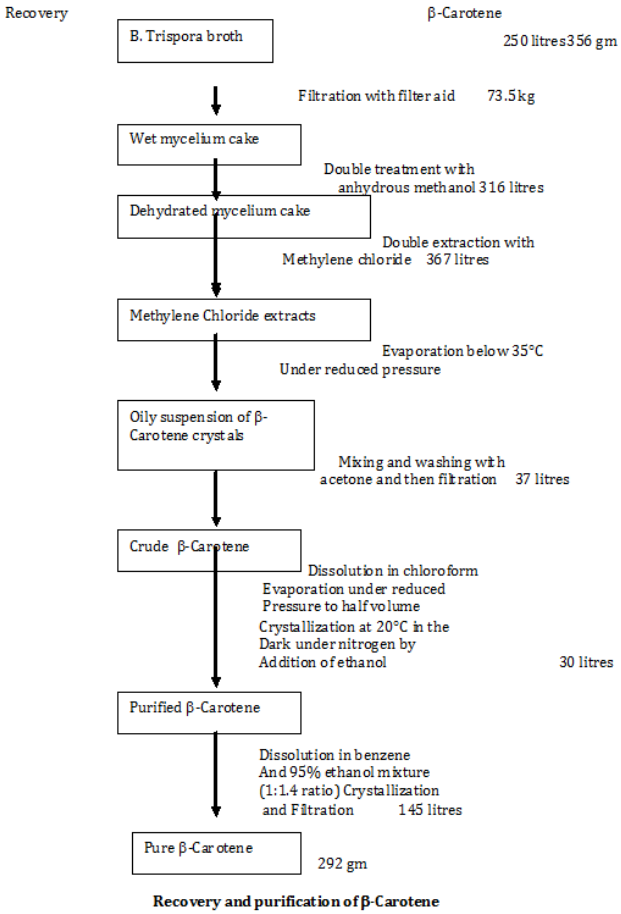
Many species of algae and fungi (e.g. *Neurospora crassa, Penicillium sclerotium, Phycomyces blakesleeanus*) and also yeasts (Rhodotorula) were considered for use in β-Carotene production but were found unsuitable. Some particular fungi in mucorales group and choanophoraceae family of Phycomyces concentrated the interest on the development of industrial fermentation. Particularly Blakeslea trispora have received extensive study for their ability to produce β-Carotene. This microorganism is heterothallic in nature. High concentrations of β-Carotene are produced only when both the mating types are present in the medium.

Growth conditions

The medium should be viscous and rich in vegetable oils, kerosene and surface-active agents, β or β- ionones are added during the incubation.

Activators of β-carotene production

β-Ionone, a precursor of β-Carotene is not directly incorporated into the β-Carotene but it activates the enzymes required for carotene production. β-Carotene production can also be enhanced by the presence of dimethyl formamide, α-pyrrolidone and succinimide in addition to β-ionones. **(Flow Chart 15)**



Recovery and purification of β-Carotene

Flow Chart 15

Recovery and Purification of β-Carotene

Crystallization

It is the formation of solid particles within a homogenous phase. It may occur as the formation of solid particles in a vapor, as solidification from a liquid melt or as crystallization from liquid solution. Crystallization from solution is important industrially because of the variety of materials that are marketed are in crystalline form. In industrial crystallization from solution, the two-phase mixture of mother liquor and crystals of cell sizes that occupy the crystallizer and withdrawn as product is called Magma. Crystals have been classified into seven classes these are: cubic, hexagonal, trigonal, tetragonal, orthorhombic, monoclinic, and triclinic. A given material may crystallize in two or more different classes depending upon the conditions or crystallization e.g. calcium carbonate occur commonly in nature in hexagonal form (as calcite) but also it occurs in the orthorhombic form (as aragonite). Under ideal conditions, a growing crystal maintains geometric similarity during growth; such a crystal is called invariant. Unless the crystal is a regular polyhedron,

the rates of growth of various faces of an invariant crystal are not equal.

Principles of crystallization

Crystallization may be analyzed from the standpoint of purity, yield, energy requirements, rates of nucleation and growth. Purity- crystals are purified from the mother liquor by filtration, centrifugation and then washing the crystals with fresh solvent. The effectiveness of these purification steps depends upon the size and uniformity of the crystals. Equilibrium- Equilibrium in crystallization process is reached when the solution is saturated and equilibrium relationship for bulk crystals is the solubility curve. Yields- In many industrial crystallization processes, the crystals and mother liquor are in contact long enough to reach equilibrium, and the mother liquor is saturated at the final temperature of the process. The yield of the process can then be calculated from the concentration of the original solution and the solubility at the final temperature. Supersaturation- In the formation of a crystal two steps are required 1) the birth of a new particle 2) its growth to macroscopic size. The first step is called Nucleation.

Types of crystallizers

Commercial Crystallizers are different in several ways. The difference lies in how the crystals are brought into contact with supersaturated liquid. The first technique called the 'Circulated liquid method', a stream of supersaturated solution is passed through a fluidized bed of growing crystals, within which supersaturation is released by nucleation and growth. The saturated liquid then is pumped through a cooling or evaporating zone, in which supersaturation is generated and finally the supersaturated solution is recycled through crystallizing zone. In the second technique called 'Circulating Magma method', the entire magma is circulated through both crystallization and supersaturation steps without separating the liquid from solid. Supersaturation as well as crystallization occurs in the presence of crystals. In both the methods feed solution is added to the circulating stream between crystallizing and supersaturating zone.

Precipitation

Precipitation phenomenon is used to obtain products from the broth or some times, to remove impurities from the ongoing fermentation process. There are a number of processes where insoluble precipitates are isolated. Since organic solutes have solubilities dependent on the solution temperature, pH, composition, ionic strength and dielectric constant therefore precipitation can be brought about in many ways as follows:

1) By adding precipitant to react with solute and producing an insoluble product, often a salt e.g. procaine hydrochloride + penicillin β Procaine-penicillin.

Organic solvent + streptomycin + sulfuric acid β Dihydrostreptomycin sulfate

Organic solvent + Erythromycin + water β Erythromycin hydrate

Biopolymer recovery is also obtainable by salt addition e.g. Xanthan gum is a polyanion and calcium ion can be used to form gel precipitate. Alginate biopolymer is recoverable from algal biomass by cell removal (filtration), followed by calcium chloride precipitation of the biopolymer.

2) Solvent driven precipitations are useful in the production of microbial biopolysaccharides including dextrans and xanthan gums. The biogum fermentations are typically aerobic and produce a highly viscous final broth with xanthan production, final broth pasteurization kills Xanthomonas cells. After adding KCl and then methanol or isopropyl alcohol the gum polysaccharide directly precipitates out. Dextran recovery is achieved by alcohol or acetone precipitation. In solvent driven precipitation for the production of bulk polysaccharide, the modest product value requires efficient recovery and reuse of solvent as well as good solvent removal for food or pharmaceutical grade product. 3) Protein precipitation techniques- The techniques result in a phase change to form a precipitate, require some alteration of protein solution conditions to render the original, thermodynamically stable one phase system unstable with respect to precipitation. The various methods for causing the needed reduction in solubility of protein include: 1) Added high salt concentration to give precipitates by salting out 2) pH adjustments to protein's pH of neutral charge, the isoelectric point, at which point the protein has minimum solubility 3) Reduction of medium dielectric constant to enhance electrostatic interaction by e.g. addition of miscible organic solvent 4) Addition of non-ionic polymers that reduce the amount of water available for protein solvation 5) Addition of polyvalent metal ion to form reversibly a protein precipitate. The method of choice includes considerations not only of the protein concentration needed and cost of separation technique, but also the purity of the final product compared to precipitating agent **(Table 2)**.

Recovery and purification of microbial products

When biosynthesis of products in a fermentor takes place this becomes necessary to isolate the product and convert it into a form suitable for the required purpose. The isolation procedures differ considerably depending upon the location of the product i.e. intracellular or extracellular and also depend upon the concentration and stability of the product. Sometimes microorganisms also produce many other organic products apart from the main product that may complicate the process of isolation. The simplest situation is the isolation of microbial cells when they represent the desired end product. The basic process to isolate the microbial products is shown in the following figure: **(Flow Chart 15)**

After cultivation, the culture fluid is usually processed in order to facilitate the separation of microorganisms. The treatment depends upon the composition of the fluid, the

type of cultured microorganisms and the product; it may include an adjustment of pH, heating and /or addition of substances that coagulate the microbial cells. After such treatments the microorganisms are separated by filtration or centrifugation. Further treatments depend on whether the product is intracellular or extracellular in the supernatant. The microorganisms themselves, or the filtrate, may, after suitable processing by pressing, evaporation, and etc. constitute the end product. If the product is contained in the cells, it is necessary first to disintegrate them; the method of disintegration again depends upon the type of microorganism, physiological state and composition of the cell wall. Following disintegration, the cell walls are separated and product is isolated by the methods shown in the above figure. Products contained in the supernatant are isolated-depending on their chemical properties-by precipitation, extraction, adsorption, dialysis, ultrafiltration, evaporation etc.; these methods are often used in combination. After isolation, the products are further processed to a form they are to be used for the said purpose (in medicine, food industry, agriculture, etc.) **(Table 3)**

Table 3

Parameter	Grape Juice (mls)	Fermenting Wine		
		7 days	14 days	21 days
% Tartaric acid				
% Acetic acid				
Alcohol				
Taste				
Aroma				
Clarity				

Mechanical separation of microorganisms

The choice of the method of mechanical separation and the appropriate equipment depend on the type of microorganism (Bacteria, yeast, actinomycetes or filamentous fungi), composition of the medium (synthetic or complex) and the absence or presence of suspended particles. Basically, the separation of microorganisms is carried out using one or two main methods, filtration and centrifugation.

Filtration

It may be defined as the separation of suspended particles from a liquid by means of a pressure difference through a permeable partition. The diameters of the particles may be smaller than the openings in the filter so that initially they pass through these openings. When, however, the filter pores become clogged with the particles, the filter begins to retain further particles almost completely. As filtration proceeds the thickness of the particle cake on the filter increases and the flow rate of the filtrate decreases. The gradual decrease in flow rate is caused partly by clogging of the pores on the filter surface and partly also by the diminishing distance between particles inside the pores. For rigid or non-compressible particles the filter cake may be considered as a system comprising a large number of capillary channels through which the fluid flows according to Poiseuille's law. Then

$$u = \frac{dV}{A\,dt} = \frac{K'\,\Delta P}{\mu L}$$

Where u = linear flow rate; V= filtrate volume; T= time; P= pressure drop through filter cake; L= thickness of filter cake; A= filter area; and = viscosity of fluid

$$\frac{dV}{dt} = \frac{K'A\,\Delta P}{\mu L}$$

$$\alpha = \frac{1}{K'} = \frac{A\,\Delta P}{\mu L} \div \frac{dV}{dt}$$

Food Fermentation Practical (1) -To study the wine production by the fermentative activity of Yeast cells.

I. **Principle**

Wine is a product of the natural fermentation of the juices of grapes and other fruits such as peaches, pears, plums, and apples by the action of yeast cells. This biochemical conversion of juice to wine occurs when the yeast cells enzymatically degrade the fruit sugars, fructose and glucose firstly into acetaldehyde and then into alcohol. Grapes containing 20-30% sugar contents will yield wines with an alcohol content of 10-15%. Also present in grapes are acids and minerals whose concentration are increased in the finished product and are responsible for the characteristic taste and bouquet of different wines. For red wines crushed grapes must be fermented with their skins to allow extraction of their color into juices while white wines are produced from the juice of white grapes without skins. The commercial production of wine is a long and exacting process. First the grapes are crushed to express juice called "must". Potassium metabisulfite is added to the must to retard the growth of acetic acid bacteria, molds, and wild yeasts that are endogenous to grapes in the vineyard. A wine producing yeast Saccharomyces cerevisiae var ellipsoideus is used to inoculate the must that is used to inoculate the must, which is then incubated for 3-5 days under aerobic conditions at 21-32oC. This is followed by an anaerobic incubation period. The wine is then aged for a period of one to 5 years in aging tanks or wooden barrels. During this time the wine is clarified of any turbidity and formation of esters responsible for characteristic flavor are produced. The clarified product is then filtered, pasteurized at 60oC for 30 minutes and bottled.

II. **Materials required**

Fifty mls of white grape juice broth culture of Saccharomyces cerevisiae incubated for 48 hours at 25oC. Five hundred mls of pasteurized Welch commercial white grape juice. Phenolphthalein solution 1%. Sodium hydroxide 0.1N and sucrose. One litre Erlenmeyer flask, One holed rubber stopper containing a 2" glass tube plugged with cotton plug, Pan balance, Spatula, Glassine paper, 10 ml graduated cylinder, Ebulliometer, Burette and Pipettes for titration.

III. **Procedure**

Pour 500 ml of white grape juice into one litre Erlenmeyer flask. Add 20gm sucrose and 50 ml of Saccharomyces cerevisiae containing grape juice broth culture (10% starting culture). Close the flask with the stopper containing cotton plugged air vent. Incubate the wine at 25 °C. After 2nd and 4th day of incubation, add 20gm more sucrose to the fermenting wine. Now again incubate at 25oC for 21 days.

IV. **Total acidity (expressed as % Tartaric acid)**

To 10 ml of aliquot of fermenting wine, add 10ml distilled water and 5 drops of phenolphthalein indicator. Mix and titrate it with 0.1N NaOH solution. (Table 2)

$$\% \text{ Tartaric acid} = \frac{\text{Mls of Alkali} \times \text{Normality of Alkali} \times 7.5}{\text{Weight of sample (1 ml of wine = 1gm of wine)}}$$

Volatile Acidity (expressed as %Acetic acid)

$$\% \text{ Acetic acid} = \frac{\text{Mls of Alkali} \times \text{Normality of Alkali} \times 6.0}{\text{Weight of sample in grams (1ml of wine = 1gm of wine)}}$$

Theory

Milk of high SNF (10-12 or 15%) is used for the preparation of yogurt. It is different from curd in the sense that, in yogurt milk of high SNF is inoculated with pure culture of *Lactobacillus bulgaricus and Streptococcus cremoris or Streptococcus thermophilus* in the ratio of 1:1 while in curds natural flora acts as inoculum. After fermentation, the acidity of the final product is measured in terms of lactic acid (gm per 100ml of yogurt).

V. **Procedure**

Preparation of starter culture: The inoculum of Lactobacillus bulgaricus is maintained in Micro-inoculum broth of composition: Yeast extract 20g, Peptone 5g, Dextrose 10g, Potassium dihydrogen phosphate 2g, Sorbitan monooleate complex 0.1g or Tween-80 few drops, and distilled water one litre. The inoculum of Streptococci is maintained in Nutrient broth of composition: Beef extract 3g, Peptone 5g, NaCl 5g, and distilled water one litre. Cultures of both the strains are to be mixed in 1:1 ratio in Peptonized milk of composition: Skim milk powder 10g, Peptone 5g, and distilled water 100 ml; pH 6.5 sterilized at 85-95oC for 40-60 minutes; and incubated at 21-28oC for 12-18 hours till the pH reaches 4.9-5.0. The starter culture now is cooled to 5-10 °C and is kept at the same temperature until used. It is not a good idea to hold ripe starter for more than 24 hours.

VI. **Fermentation**

Weigh desired quantity of milk and adjust its SNF to 10-12% by adding whole dry milk. Add sugar at the rate of 10%. Heat it to 80-90 °C for 20 minutes. Cool the milk to 45-48 °C (for S. thermophilus) and inoculate with 5% yogurt starter culture. Mix well. Keep the milk in clean and sterile container for setting. Incubate the milk containers at 45 °C for 3-4 hours till a firm coagulum is obtained. Remove the product from incubator and keep it at 5 °C till it is consumed.

Calculation of Milk Solid Non Fat (MSNF)

Milk at a temperature of 60oF is added up to the brim of the cylinder and lactometer is gently dropped into it. Reading on the lactometer is noted down. This reading is corrected as follows:

- ✓ Add one for every 10oF rise in temperature
- ✓ Add 0.5 for upper meniscus of the milk

This now is called as Corrected lactometer reading (CLR). The MSNF is calculated by the formula as: MSNF = CLR/4 + 0.2 × Fat + 0.14

E.g. If lactometer reading observed at 70oF comes out to be 27 then CLR is equal to 27+1+0.5 (for meniscus) = 28.5 and MSNF = 28.5/4 + 0.2× Fat + 0.14

Calculation of Acidity in Yogurt

Take one gram of yogurt in titration flask and dilute it with 5 ml of distilled water. Add to it a few drops of phenolphthalein indicator. The solution is colorless. Now add from the burette 0.1N NaOH solution drop wise till the color of solution changes to light pink. This is the end point. Repeat the experiment till a concordant set of three readings is obtained.

General Calculations

Take one gram of yogurt in titration flask and dilute it with 5 ml of distilled water. Add to it a few drops of phenolphthalein indicator. The solution is colorless. Now add from the burette 0.1N NaOH solution drop wise till the color of solution changes to light pink. This is the end point. Repeat the experiment till a concordant set of three readings is obtained.

VII. **Theory**

Natural microflora present on cabbage produces lactic acid from carbohydrates. In due course of time, after the accumulation of lactic acid to certain extent, all proteolytic and other microorganisms are eliminated from the product except lactic acid tolerant Lactobacilli. These bacteria further produce more lactic acid resulting in lowering of the pH of the product significantly after 3-4 weeks. Because of this lowering in pH other organisms do not find any access to grow in the same product.

$$\% \text{ Acidity} = \frac{\text{ML of Alkali used} \times \text{Normality of Alkali} \times 0.09}{\text{Volume of sample}}$$

(In terms of Lactic acid gm/ml)

0.09 is gram equivalence of lactic acid per ml.

Production Chart of Yogurt

Quantity of milk used _____kg Quality of fresh/ old/ pasteurized milk _____

Fat _____% MSNF_____% Acidity of milk_____%

Temperature of Heating ___ Quantity of milk used _____kg Quality of fresh/ old/ pasteurized milk _____

Fat _____% MSNF_____% Acidity of milk_____%

Temperature of Heating ___°C Temperature of Holding ___°C Temperature of Cooling____°C

Starter used_____Quantity of starter used_____

Texture_____Time of incubation_____ Quality of Yogurt_____Good/fair /Poor

Acidity of Yogurt _____gm/100ml Temperature of Storage_____°C.

Temperature of Holding ___°C Temperature of Cooling____°C

Starter used_____Quantity of starter used_____

Texture_____Time of incubation_____ Quality of Yogurt_____Good/fair /Poor

Acidity of Yogurt _____gm/100ml Temperature of Storage_____ Quantity of milk used _____kg

Quality of fresh/ old/ pasteurized milk _____

Fat _____% MSNF_____% Acidity of milk_____%

Temperature of Heating ___°C Temperature of Holding ___°C Temperature of Cooling____°C

Starter used_____Quantity of starter used_____

Texture_____Time of incubation_____ Quality of Yogurt_____Good/fair /Poor

Acidity of Yogurt _____gm/100ml Temperature of Storage_____°C.

VIII. **Procedure**

Wash cabbage with clean water. Remove the outer leaves. These leaves are kept aside for their further use. Remove the case and other undesirable area. Prepare lots of cabbage weighing ½ kg each and slice them into shredding or small pieces of 0.16-0.08 cm in thickness. Weigh the salt (2.25%) i.e. 11.25 gm for ½ kg of cabbage shredding. Put the outer leaves that were kept aside, at the bottom of a glass jar. Take one lot of shredded cabbage and layer onto the leaves inside the glass jar. Sprinkle ¼th of the salt on the cabbage and wait for its absorption in the shredded cabbage. Similarly layer the rest of the shredded

cabbage and sprinkle the salt onto it till all the cabbage and salt finishes for the preparation of sauerkraut. The addition of salt serves two main functions. Firstly, it draws moisture out of cabbage that dissolves the salt forming a brine solution, which acts as a fermenting medium for Lactobacilli and also equally distributes them in the medium. Secondly, it inhibits the growth of proteolytic bacteria. Now place the plastic bags filled with water as a weight to press the cabbage and close the lid of the glass jar. Fermentation was carried out at 25oC for 3-4 weeks. Note down the change in pH and color after every week.

Microbiology of Sauerkraut Fermentation Pederson first described the lactic acid bacteria that he observed in fermenting sauerkraut. He found that the fermentation was initiated by the species of Leuconostoc mesenteroides. This species was followed by gas forming rods and finally by non-gas forming rods and cocci. Leuconostoc mesenteroides is a hetero fermentative bacteria and it grows more rapidly than other lactic acid bacteria. It is active over a wide range of temperature and salt concentrations. It produces acid and carbon dioxide that rapidly lowers the pH, thus inhibiting the activity of undesirable microorganisms and enzymes that may soften the shredded cabbage. The carbon dioxide replaces the air and creates an anaerobic condition favorable to prevent oxidation of ascorbic acid and natural color of the cabbage. It also stimulates the growth of many lactic acid bacteria. While this initial fermentation is developing, the hetero fermentative species of lactobacillus brevis and homofermentative species of lactobacillus plantarum and sometimes Pediococcus cerevisiae begin to grow rapidly and contribute to the major end products like lactic acid, carbon dioxide, ethanol, acetic acid. Minor products also appear in the fermentation. The minor products are a variety of volatile compounds e.g. diacetyls, acetaldehyde and primary carbonyls.

Role of temperature in sauerkraut fermentation

Lower the initial temperature better is the product formation. It is considered that the initial temperature of 18.3oC produces superior quality sauerkraut because at lower temperature hetero fermentative lactic acid bacteria exert a greater effect.

Spoilage of Sauerkraut

Common spoilage signs found in sauerkraut are discoloration, off-flavor, off-odor caused by yeast and mold growth, loss of acidity and slimy product due to the dextran formation by Leuconostoc mesenteroides. The proteolytic activity of molds and yeasts and also by asporogenous yeasts produces product pink in color. Such type of spoilage is known as “Pink kraut”.

Food Fermentation practical (4) -Preparation of Sweet Acidophilus milk

I. **Theory**

The fermented acidophilus milk is known for its therapeutic value and has been successfully tried in cases of chronic colitis and gastro-intestinal disorders in general. It is prepared by the inoculation of pure culture of Lactobacillus acidophilus. It is a very acidic product. The acidophilus milk has not gained popularity as that of other fermented milk products because of its taste and off-flavor. When it is sweetened with sugar, it is called as sweet acidophilus milk. Sweet acidophilus milk is gaining popularity now a day.

Medium for maintenance for lactobacillus acidophilus

The organism is maintained in Tomato Juice Agar of Composition: Tomato juice 400ml equivalent to 20g tomatoes, Peptone 15g, Skimmed milk powder 10g, Agar-Agar 1.5-2.0%; pH 5.0; sterilize at 15lb pressure for 15 minutes.

Procedure for ordinary acidophilus milk

Take adequate quantity of low fat milk. Boil or steam it for 20 minutes. Cool it to a temperature of 28-30^{0}C. Pass carbon dioxide gas through it for 1-2 minutes. Inoculate it with 1-2% inoculum of Lactobacillus acidophilus. Incubate at 37-40 °C for 30-40 hours.

Procedure for sweetened acidophilus milk

Inoculate cold pasteurized sweetened low fat milk with 1-2% of pure culture of Lactobacillus acidophilus. No incubation is done. Inoculated milk is held under refrigeration at 7°C or below for 30-40 hours or till it is consumed. It tastes exactly like low fat milk.

Food Fermentation Practical (5) -Production of Lactic acid

Materials required

Pure culture of Lactobacillus delbrueckii B-70 and Production medium of composition: Sucrose 100g/L, Yeast extract 20g/L, Potassium dihydrogen orthophosphate 2.5g/L, Calcium carbonate 10%, Agar-Agar 2% (for solid medium); pH 7.0-7.2; Sterilize at 15 lb pressure for 15 minutes. Vitamin B-complex+ Aspartic acid +Folic acid combination may be used in place of yeast extract.

Medium for inoculum preparation

Inoculum is prepared in Glucose Yeast Extract medium of composition: Glucose 10%, Yeast extract 2% and Potassium dihydrogen orthophosphate 0.25%.

I. **Procedure**

Take two litres of production medium in laboratory fermenter and inoculate it with 10% of inoculum of L. delbrueckii B-70 prepared in Glucose Yeast extract medium. Incubate it at 37 °C for 5-7 days. The medium is gently stirred during the incubation period to keep the calcium carbonate in suspension.

Recovery of lactic acid

Filtration -The suspension is filtered with conventional laboratory filters. Acidification -To the filtrate add concentrated sulfuric acid to form precipitates of calcium sulfate. Filter and wash the precipitates with water. The washings are added into the filtrate. Removal of Impurities -The filtrate is treated with activated charcoal to remove the impurities. Filter again. Concentration –The filtrate is evaporated on a steam bath to concentrate it to 25% solids and then again subjected to evaporation till 50% solids are obtained. Removal of Heavy metals -The heavy metals like lead is removed by adding sodium or potassium ferrocyanide into the concentrated mass and filtering it. The filtrate contains lactic acid, which can be purified by passing it through ion exchange resin column. The lactic acid so obtained may have 93-95% purity.

Food Fermentation Practical (6) –Production of Amylase enzyme and its estimation

Materials required

Inoculum -An Amylase producing strain of Streptomyces spp. preserved in Nutrient medium of composition: Beef extract 3g, Peptone 5g, NaCl 5g, and distilled water one litre. Calcium chloride solution 1% in water, Starch solution 0.1% in 0.05M acetate buffer pH 5.2 (dissolve 1mg starch in 1ml of acetate buffer), Production medium of composition: Beef extract 3g, Peptone 5g, NaCl 5g, and distilled water one litre. Add starch 10g per litre into the nutrient broth medium. Sodium acetate-Acetic acid buffer solution (0.05M) -Sodium acetate 2.72g dissolve in 100ml distilled water (solution-I), Acetic acid 1.15g dissolve in 100ml distilled water (solution-II); add solution-I into solution-II and adjust pH to 5.2, make volume 100ml and heat it to 60oC for 10-15 minutes.

I. Procedure

Dispense 100ml of production medium in two 250ml conical flasks and sterilize it at 15lb pressure for 15 minutes. Inoculate the flasks with Streptomyces spp. culture that was preserved in nutrient broth medium at the rate of 5%. Incubate the flaks on shaker at 37 °C for 96 hours.

Standard curve for starch

Prepare dilutions of starch in acetate buffer from 0.1% to 0.01%. To each of these dilutions, add 0.2ml Iodine solution and add water to dilute it to 10ml. Measure the optical density at 520nm. For control take 1ml of distilled water in place of starch dilution and to this add iodine solution; observe optical density in a similar manner.

Estimation of amylase

Prepare Control, Blank and Digest. Control -To 6ml of 0.1% starch solution, add 1ml of 1% calcium chloride, 2ml of hydrochloric acid, and 1ml of distilled water and 0.2ml of iodine solution. Measure optical density at 520nm. Blank -To 6ml of 0.1% starch solution, add 1ml of 1% calcium chloride solution, 1ml of enzyme extract, 2ml of hydrochloric acid and 2ml of iodine solution. Measure optical density at 520nm. Digest -To 6ml of 0.1% starch solution, add 1ml of 1% calcium chloride solution and 1ml of enzyme extract. This is incubated at 30 °C for 10 minutes. Now add 2ml of HCl and 0.2ml of iodine solution. Measure optical density at 52 nm.

Calculation of Enzyme activity

$$\text{Enzyme Activity (mg/minute or IU/ml)} = \frac{\text{1mg/ml Starch} \times (\text{OD Control} - \text{OD Digest})}{\text{Time of Incubation}}$$

Food Fermentation practical (7) – To study the alcoholic fermentation by Yeast

I. Theory

The yeast Saccharomyces cerevisiae converts fermentable sugars (glucose, fructose and sucrose) into ethanol and carbon dioxide. In large-scale production of alcohol, molasses is used as substrate. Blackstrap molasses contains 45-55% w/v fermentable sugar as sucrose, which is metabolized by yeast through Embden-Meyerhoff-Parnas Pathway to produce ethanol and carbon dioxide as the end products.

Materials required

Molasses; fermentation jar (10 L); micro distillation unit; Test tubes; conical flasks; Standard volumetric flasks (25ml); Urea; DNS reagent (3,5-Dinitosalicylic acid 1%, Phenol 0.2%, Sodium carbonate 0.05%, Sodium hydroxide 1% and Sodium potassium tartarate 20%); Dichromate reagent (Dissolve 34 gm of Potassium dichromate in 500 ml of distilled water, add 325 ml of concentrated Sulfuric acid slowly keeping the flask in ice bucket); YPD medium of composition: Yeast extract 10gm, Peptone 20 gm,

Dextrose 20gm and Agar-Agar 20gm; Slant culture of yeast S. cerevisiae.

I. Procedure

Preparation of Inoculum: Prepare 250ml GYE / YPD broth medium. Add 50ml in separate flasks and autoclave at 15lb pressure for 15 min. Inoculate slant culture of yeast aseptically in each 50ml flask. Incubate the flasks at 28 °C for 16-18h.

II. Inoculum for fermentation

Dilute molasses to 12 ^{o}Brix (1.1Kg molasses in 8 litre of water). Adjust pH to 5.5 with 10N Sulphuric acid. Sterilize at 10lb pressure for 30min and then cool. Inoculate this 250 ml molasses medium (2 flasks) with 10% of the culture grown in YPD. Incubate the culture in shaker for 12h at 28 °C.

III. Fermentation medium

Dilute the Black strap molasses with tap water to 22 ^{0}Brix (2.1Kg molasses in 8 litre water). Adjust pH to 5.5 with 10N Sulphuric acid. Add 200mg Sodium dihydrogen phosphate and 200mg Urea per litre respectively. Maintain pH 5.5. Sterilize medium at 10lb pressure for 30min. Inoculate with 10% v/v inoculum and incubate at 28 °C for 48-72h for fermentation. After the fermentation has ceased close the mouth of the flask with an airtight bung to provide anaerobic conditions so that alcohol production may take place. Withdraw 10ml sample at every 12h interval and estimate alcohol and sugar concentration. Plot a graph with time on X axis and alcohol and sugar concentration on Y- axis.

Alcohol estimation

Preparation of standard curve for alcohol concentration: Prepare 1-10% v/v alcohol in

Various test tubes. Take 1ml of the various concentration of alcohol into 25ml of distilled water in 100ml distillation flask fitted with Liebig condenser. Distill and collect 15ml of the distillate in a 50ml volumetric flask containing 25ml Pot. Dichromate solution. Make up the volume to 50ml. Keep the alcohol-Pot. dichromate complex at 60 °C for 30 min. Measure OD at 600nm. Plot a standard curve with concentration of alcohol on X-axis and OD at 600nm on Y-axis.

Determination of Alcohol Concentration from fermentation Medium

Take 1ml sample and mix it with 25ml of distilled water. Distill as above and measure OD at 600nm. Find the alcohol concentration from the standard graph.

A. If molasses could not be obtained for laboratory exercise, use YPD medium with 50g/L sucrose for inoculum development and with 150g/L sucrose for preparation of fermentation broth.

Food Fermentation Practical (8) -Preparation of Idli

Ingredients: Idli preparation contains a number of different ingredients these are 1) Rice 2) Black gram dhal 3) Salt 4) water. Dehulled soybeans or Bengal gram can be used as a substitute for black gram dhal and a number of cereal grains can replace rice. However, there may be marked change in the texture and flavor when using substituted materials. It has been reported that rice variety and its physical characteristics are very important to produce a good quality idli. White Kar and IR20 varieties of rice have given much better performance in the production of idli, especially the White Kar variety because of its high amylose content, low amylopectin content better gelatinization, and better water uptake ability.

Proportion of cereal to legume: Ordinary idli consists of three parts rice and one part Black gram dhal plus salt to taste. Kancheepuram idli is prepared from one part rice and one part black gram dhal plus cashew nuts, ghee, salt, pepper, ginger and cumin added to taste. Normally a proportion of rice to black gram dhal varies from 4:1 to 1:4, the 2:1 being the best. It has been seen that when black gram dhal proportion is less than 25%, the steamed idli was hard and organoleptically unacceptable whereas when it is more than 50%, the product obtained is too sticky to be acceptable. Thus, not only can the ingredients be varied, but the proportions can also be varied within a wide range and still an acceptable product is obtained.

I. Procedure

White polished rice is carefully washed and soaked for 5-10 hours. Black gram dhal is carefully washed and soaked for 5-10 hours. The rice is then drained and coarsely ground in a stone mortar or other grinder. The black gram dhal is drained and finely ground in a stone mortar. The rice and black gram dhal slurries are combined to form a rather thick batter, which is stirred with hands. Salt is added to taste. Other seasonings, such as chilies are occasionally added. The batter is placed in a warm place to ferment overnight. In the morning, the batter is poured into the cups of an idli steamer, which is placed in a covered pan or cooker and steamed until the starch is gelatinized and the idli cakes are soft and spongy.

Food Fermentation Practical (9) -Preparation of Dosa

II. Ingredients

The ingredients used for dosa preparation are similar to as that of idli preparation i.e. rice, black gram dhal, salt and water. Rice may be substituted by wheat, bajra *Pennisetum typhoideum*, maize, or kodri and black gram dhal may be substituted by sprouted peas, cowpeas (Vigna catjang), field beans (Dolichos lablab) or soybeans. Fresh groundnut oilcakes may also be substituted for black gram dhal.

III. Soaking and batter formation

Generally, equal quantities of rice and dehulled black gram dhal are soaked in water at room temperature separately for 5-10 hours. It is common practice that finely ground powders are used to prepare batter. The finely ground powders are mixed with water at temperatures

ranging from 48° to 98 °C, best at 80 °C for the preparation of batter. Water is added in the range of 2.0 to 2.2 times the initial dry weight of ingredients to prepare a batter of viscosity desired for dosa. Salt is added from 0.8 to 1.0% as a seasoning before fermentation.

IV. Fermentation time

Traditionally, dosa batter is kept overnight for fermentation. The fermentation time should be sufficient to allow a definite leavening and acidification of the batter and to allow for the development of a pleasant acid flavor.

V. Incubation temperature

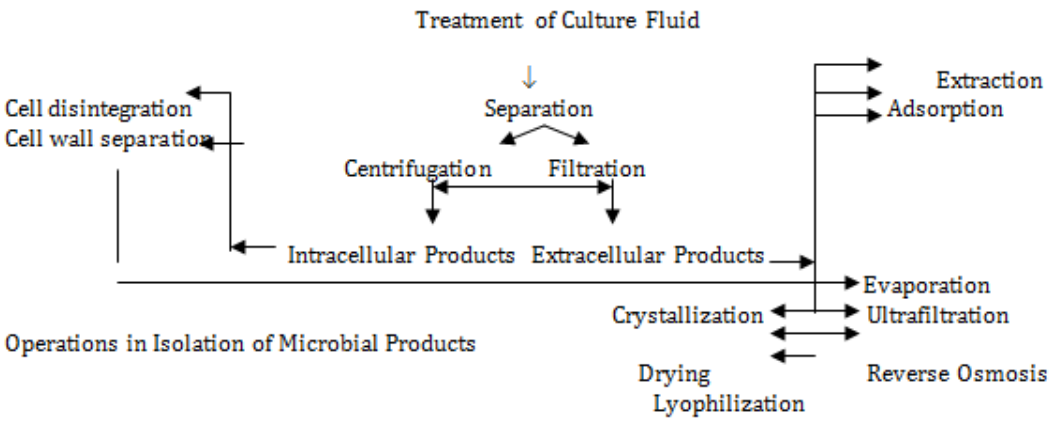
Ordinarily, the dosa fermentation is carried out at room temperature. In the tropics, this generally means a temperature of 25-30 °C.

VI. Harvesting and preservation

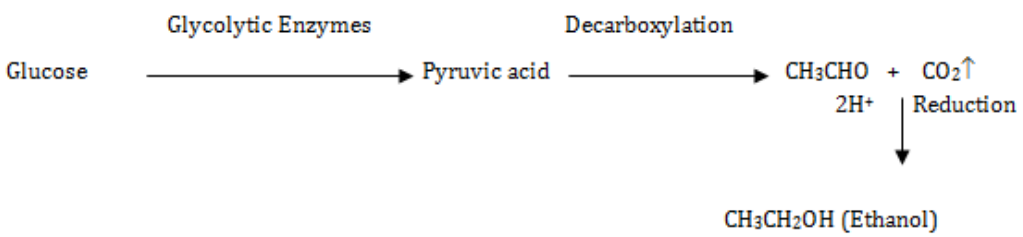
As soon as the batter becomes leavened and acidified, it is spread onto a hot and greasy griddle where it assumes the shape of a crisp pancake. The spreading of dosa on the griddle is an art that matures with practice. Sometimes a mixture of cooked different vegetable is poured onto the crisp dosa and the sides are rolled over it, which now becomes ready to be eaten with a spiced soup of dhal and vegetables popularly known as Samber. Food Fermentation Practical (10) -Production of Citric acid by *Aspergillus niger* in media containing molasses and sucrose.

VII. Theory

Many microorganisms like molds (*Aspergillus niger, A. awamori, Penicillium janthinallum, Trichoderma viridae, Mucor piriformis,* etc.), yeasts (*Candida lipolytica, C.tropicalis, C.citrica, Hansenula, Rodotorula, Pichia, Torulopsis* etc.) and bacteria (Bacillus licheniformis, Bacillus subtilis, Brevibacterium flavus, Corynebacterium species) have the capability to produce citric acid. Commercially spores of Aspergillus niger are employed to produce citric acid. It accumulates in culture solutions of pH 1.8-2.0. In fungi different metabolic pathways are involved in the production of citric acid but 78% of the citric acid is produced by the involvement of glycolysis and Tricarboxylic acid cycle. The acetyl COA derived by EMP pathway condenses with Oxaloacetate of Kreb's cycle to produce citric acid. Citric acid is an important chemical used in medicines, flavoring extracts, food and candies and in dyeing industry.



Flow Chart 16



VIII. Materials required

Molasses; Sucrose; Flasks; Test tubes; Sodium hydroxide; Ammonium dihydrogen orthophosphate; Trace element solution of composition: Zinc sulfate 3mg/100ml, Copper sulfate 680mg/100ml, Magnesium sulfate 2.0g/100ml and EDTA 2.0g/100ml; Dissolve in 100ml distilled water and incubate at 25oC for 3 days.

Composition of production medium of citric acid

Molasses or Sucrose 45gm/300ml, Ammonium dihydrogen orthophosphate 0.75gm/300ml, Potassium dihydrogen orthophosphate 0.3gm/300ml, Tween-80 0.6gm/300ml; pH for molasses 5.0-6.0 and for sucrose 2.0-3.0 Add trace element solution before sterilization. Sterilize at 15lb pressure for 15 minutes.

I. Procedure

Prepare two sets (one for molasses and second for sucrose) of 250ml flasks. Each set having three flasks containing 100ml production medium. One set having molasses and sucrose in the second set. Label the flasks of each set as control flask, Methanol containing flask, and flask without methanol. Add 3ml of methanol in the flask labeled as methanol flask. Sterilize both the sets at 15 lb pressure for 15 minutes. Cool the flasks and inoculate all the flasks of both the sets with spores of *Aspergillus niger* except the control flask. Incubate the flasks at 25 °C for 4-5 days on shaker. After the fermentation is over, filter the contents. Compare the acidity of the filtrate with the control flask.

II. Acidity

Titrate the filtrate against 0.1N NaOH using phenolphthalein as an indicator. Appearance of pink color is the end point. Note down the volume of filtrate used in titration and calculate the strength of citric acid in filtrate.

General calculations

NaOH Filtrate (citric acid)

$$N_1 \times V_1 = N_2 \times V_2$$

$$0.1 \times 10 = N_2 \times B \text{ (say); } N_2 = 1/B; \text{ B = Volume of filtrate used}$$

$$\text{Strength of citric acid} = \text{Equivalent weight of citric acid} \times \text{Normality}$$

$$= 64.3 \times N_2 = \text{'Z' (say).}$$

Basic of Fermentation Technology

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Contents

Origin and Evolution of Fermentation Process and Fermented Foods

In earliest times, man was plagued with either feast or famine, so any means he could discover to conserve food when it was plenty was a great step forwarded in his survival and his conquest of earth. Since man was of necessity a wanderer and a hunter, he learned about the drying and smoking of meat. Certainly these methods not only conserved his supplies, but they also reduced weight, enabling him to carry more food with him. At the times of discovery of North America by Europeans, most of the Indians were in this stage of development. The discovery of two methods Drying and Smoking just like the invention of wheel perhaps took place by serendipity. Early man might not know why foods spoil he knew they did. Later we can speculate that he discovered the use of salt with drying and smoking.

Man's next discovery for preserving food was the fermentation of foods, although he had no idea what happened when microbial growth occurred, he learned that plant materials and meat could be kept for long periods of time when they have undergone fermentation. It was also essential that he knew how to use salt (a necessary agent that inhibits toxin production from microorganisms in a successful fermentation). Undoubtedly many an early ancestors of man died from botulism or was made ill by Staphylococcus aureus. After the addition of salt in natural fermentation there he knew definite changes in color, odor appearance and taste, which helped the product to be wholesome. Probably the first fermentation was discovered accidentally when salt might have selected certain harmless microorganisms that fermented the product to give nutritious and acceptable food. If we speculate along these lines, we might expect the first fermented food have been fish. With the advent of certain religions in which meat was excluded from diet, the use of salt and fermentation was adapted to certain plant products. For instance Bush (1959) states that Buddhism was well established religion in China and Korea by 4th century. It was introduced to Japan between 500-600 AD. It may very well be the cultivation of Soya beans and their use in food including fermented foods were then introduced in Japan. For centuries Balkan people have enjoyed fermented milk or yogurt and central Asian tribesmen have found equal pleasures in sour camel's milk or Koumiss. The ancient Sanskrit scriptures of India, the Vedas, documented the food value of Dahi- a fermented milk product similar to yogurt. Further evidences for the existence of soured milk as a food in the early times can be found in Bible. The historical, geographical, ecological and dietetic patterns in various regions of the world are reflected in diversity, variety and types of fermented milks in vogue today. These products are generally produced by the intense activity of the Lactic acid bacterial cultures.

Bread that has been known almost as long as agriculture itself, its preparation involves a yeast fermentation. Loaves of bread have been found in Egyptian pyramids built in six thousand years ago. The art of fermented doughs from cereals was practiced before recorded history. This and the production of liquid, fermented mashes from cereals are closely related processes. It is likely that liquid from a fermented mash was drunk as a slightly alcoholic beverage, while semisolid mash was needed into dough and baked. Even today yeast strain used in the production of ale and bread is that from single species of *Saccharomyces cerevisiae.* Until into the middle of the 19th century bakers obtain their yeast from breweries. At that time lager beer strains of *Saccharomyces uvarum (S. carlsbergensis)* were introduced into central Europe and later in the United States. These strains tolerate high osmotic pressure in the dough and bakers were forced to look for another source of yeast. Distiller's yeast that are also strains of *Saccharomyces cerevisiae* perform reasonably well in bread making, but they were difficult to separate from the distilling mash. This led to the establishment of a separate industry that produced baker's yeast on commercial scale for sale to bakers and for home baking. The production of baker's yeast was increased many folds with the advent of Fed-batch culture.

The discovery of fruit fermentation was made so long ago that the ancient Greek believed wine had been invented by one of their gods, Dionysus. The manufacture of wine have been recorded about 3500 B.C. as wine industry of Fertile Crescent that spread west (around the Mediterranean), North (to Hungry, Germany and France) and in the post Columbus period to America, South Africa and Oceania. Romans advanced the art of wine making, but it was an industry of large risks due to spoilage until the mid nineteenth century. The research of Louis Pasteur revolutionized the wine industry. A Mesopotamian clay tablet written in Sumerian-Akkadian about 500 B.C. tells that brewing was an established profession 1500 years earlier. An Assyrian tablet of 2500 B.C. lists beer among commodities that Noah took abroad his ark. Egyptian documents dating back to 4th dynasty about 2500 B.C. describes malting of barley and the fermentation of beer. Kui- a Chinese rice beer has been traced back to 2300 B.C. When Columbus landed America, he found that Indians drank beer made from Corn. According to Weeks (1949) the etymology of word "beer", as we know today indicates it originated from Latin verb 'bibre' (means to drink). Similarly, the Spanish word for beer 'Cervaza' apparently originated from cervisia, which combines Latin word 'Ceres' (goddess of grain) and 'Vis' (vigor). The art of brewing was spread to England by Teutons that settled in Rhine area became Germanic tribe. The major brewing centers were eventually established in Pilsen, Czechoslovakia, Munich, Dortmund, Germany, Burton-O-Trent, England, Dublin and Ireland. American Indians were already making beer from maize but Mayflower Company brought English type beer to America. English Ale beer was used till 1840s, but German Lager beer became more accepted type of beer because of its superior keeping quality.

The process of fermenting sausages was probably one of the earliest forms of meat processing and its manufacturing probably began before written history. The first mention of written history was in 9th century B.C. when it was mentioned in "Homer's Odyssey". The sausage was

called as “Oryae”. The word “Salami” was coined from the product made in Salamis- a Cyprus city destroyed in 449 BC (Pederson, 1979). Sausages eaten by Babylonians, Greeks and Romans were no doubt fermented and dried meat products. Brested (1938) stated that “Caeser’s legions” in Gaul consumed dry sausages. The descriptions of the process of making sausages confirm that Babylonians, Greeks and Romans ate many types of dry sausages. The various regions of Mediterranean developed characteristics sausages e.g. Salamis developed Genoa, Milano and Lambardi types of sausages (Anon, 1938) The Mediterranean countries consumed a highly seasoned non-smoked products classified as Latin type. Non-Europeans countries developed a Roman product, but slightly spiced, heavily smoked, moist and higher in salt content. This product is often referred to as Germanic type. In colder areas sausages are made in the winter months, stored and aged until summer, hence they are called as Summer Sausages. The aging occurs by indigenous flora prompting the growth of Lactic acid bacteria, yeasts and molds in and on the surface of sausages. Early in 20th century bacteria were discovered to be responsible for Lactic acid production and nitrate reduction in sausages. Further research in the microbiology has led to the production of very safe processed meat products and newer products are under the stage of development. Many fermented products have been proved to possess some medicinal values.

Development of Fermentation Process and Industry

Development of fermentation process may be represented by five overlapping stages. Stage I represents the pre-1900 development that is confined to potable alcohol and vinegar. Wooden vats and even fitted with some process control like thermometers (1757) and primitive heat exchangers (1801) replaced the ancient traditional Beer production by Egyptians. In mid 1800s Cagniard-Latour, Schwann and Kutzing demonstrated role of yeast in alcoholic fermentation independently. Pasteur later convinced that pure culture of these microorganisms produces more alcohol than the mixed culture. Methods for isolating and propagating pure yeast cultures were developed in the late 1800s. By the late 1800s and early 1900 generator for the production of vinegar was developed, which was considered as the first aerobic fermentor to be developed. In this method 10% good vinegar was added to the medium as an inoculum that also makes the medium acidic to make it contamination free. Thus in the beginning of 20th century concepts of process control for the fermentation process were developed.

Between the years 1900-1940 the main thrust areas of research were baker’s yeast and organic solvent fermentations. Newer products developed were yeast biomass, glycerol, citric acid, lactic acid and acetone-butanol. Studies indicated that growth of yeast in the fermentation broth leads to oxygen depletion, which results in the ethanol production at the expense of cell formation. Adding more broth in the previous broth can regulate the subsequent growth of yeast cells. This technique now is called as “Fed-batch Culture. Further studies also showed that growth of yeast cells could be improved by sparging air in the fermentation broth. During the First World War Weizmann introduced a concept of Aseptic fermentation the development of Acetone-Butanol fermentation. Steam sterilized hemispherical topped and bottomed vertical steel cylinders were used as fermentors. These fermentors had problems of inoculum development and maintenance of aseptic conditions. In spite of all the hindrances these organic fermentations paved a way for the introduction of aseptic aerobic fermentation technology.

In the Third Stage Penicillin fermentation process was developed, which was a wartime need. This fermentation was very vulnerable to contamination. All the knowledge gained in the previous year’s regarding process control, air sparging, isolation and propagation etc. were applied for the synthesis of Penicillin at a large scale. The development of large-scale extraction process and initiation of strain improvement programme was advancement at that time. Many other fermentation processes were developed at that time like antibiotics, vitamins, gibberellins, amino acids, enzymes and steroid transformations. The fourth Stage (early 1960s) is marked by the production of microbial biomass as a source of feed proteins. Many waste products were considered as a carbon source for the development of microbial biomass. Hydrocarbons as another potential source for carbon for microorganisms were discovered. In this period Jet and Pressure cycle fermentors were developed that eliminated the need of mechanical stirring. Other advantages of these processes were that they can be run continuous and were economic. At this time Batch and Fed batch culture techniques were common in the industry, but their application became short lived because of the development of Continuous Culture. The high standards of the aseptic operation and process controls were achieved by the introduction of computer systems in the fermentation process to minimize the possibility of human error. The fifth Stage in the progress of fermentation process is the introduction of Genetic Engineering and Recombinant DNA technology in the strain improvement programme. These Techniques not only allowed the transfer of genes between unrelated organisms but also enable the extremely precise alteration of genome of a particular organism. The development of further stages in fermentation will depend upon the new advances in this area.

Fermented Foods

Fermented foods form an important part of human diet. Fermented legume and cereal products are especially popular in South East Asia including India, Middle East and Africa. Traditional fermented foods are important elements in the diets of millions of people of particularly in developing countries and the methods for their preparation are simple and inexpensive. Indigenous fermented foods are so prepared that they utilize cheap sources, supply proteins, and enrich starchy diets with vitamins and other nutrients. The exact origin of fermented foods is not known and their

discovery is considered to be purely by chance. The Asians centuries ago knew the art to produce meat like flavors from vegetable proteins. The Indonesians had various methods to introduce meat like texture into the vegetable products. Such foods have a particular place in their diets. Koreans introduced acid fermented vegetables and People of Egypt developed Bread leavened with yeasts while Indians discovered methods for souring and leavening cereal-legume batters. Nearly every nation in the world has one or more fermented milks. Fermented milks are used to restore the natural flora of intestine impaired by disease or antibiotic activity. In many countries cultured milks are widely promoted and credited with health giving properties. Yogurt, Kefir, Acidophilus milk, Bulgarian milk, and Koumiss are a few names that are very popular in many parts of the world and even in western countries. Much interest over the years has been generated in the fermented foods of Asian and African countries because such foods in these countries are prepared traditionally, using simpler technology and equipments. In India preparation of fermented foods has gained a status of small-scale cottage industry that manufactures such foods utilizing natural microflora from staples and surroundings.

Products of Fermentation

A variety of products can be obtained by fermenting different substrates with the help of microorganisms. Fermentation products are the primary or secondary metabolic products of microorganisms that are produced at certain stage of their life cycle. Primary products are Growth Associated products, their concentration in the medium increases, as microorganism grows in the medium i.e. concentration increases gradually in the exponential growth phase of microorganism. Secondary products are Non-Growth Associated products and are produced in the stationary phase of growth of microorganism. The concentration of Non-growth associated products may sometimes become toxic to the microorganism itself e.g. Antibiotics.

Kinetics of Growth Linked Product Formation

The formation of growth Associated product may be described by the equation:

$$dp / dt = qp.x \quad (1)$$

Where p is the concentration of product and qp is the specific rate of product formation. Also the product formation is related to biomass production by the equation as:

$$dp / dx = Yp / x \quad (2)$$

Where Yp/x is the yield of product in terms of substrate consumed.

Multiplying equation (2) on both sides by dx/dt we get:

$$dp / dt = Y_p / x \quad \text{----------(i)}$$

$$dp / dx = Y_p / x \quad \text{---------(ii)}$$

Therefore:

$$dp / dt = Y_p / x \,.\mu \,. x \quad \text{----------(iii)}$$

Combining equations (i) and (iii) we have

$$Q_p = Y_p / x.m$$

It may be seen that when product formation is growth associated the specific rate of product formation increases with specific growth rate. When product is Non-growth associated the specific rate of product formation may remain constant over a wide range of growth rates or it may vary in a complex manner. Garden relates the formation of products to substrate utilization or in other way this classification assesses the extent to which the energy producing reactions are coupled to the product forming reactions. This approach is now very much used in studying the continuous process. According to Gaden's Classification product forming reactions fall into following three categories:

Type -I, Type -II and Type III

Type I arise as a result of primary energy metabolism. The desired product results from a carbohydrate substrate e.g. glucose to ethanol, glucose to lactic acid etc. The metabolic roots are serial with μF negative. The kinetic approximation of alcohol fermentation is given by:

$$\frac{d(\text{product})}{dt} = -k_1 \cdot \frac{d(\text{substrate})}{dt} = k_2 \cdot \frac{d(\text{cell mass})}{dt}$$

Negative sign indicates that substrate is decreasing.

In Type II the main product arises from energy metabolism but indirectly e.g. Citric acid fermentation, some amino acid fermentation. The reaction patterns are complex and restricted or abnormal metabolism is involved. The overall free energy change is negative. Such types of products are also called as Intermediate metabolites. Gaden suggests following prototype reaction for the complex dissimilation of

Type II metabolites: (Figure 1)

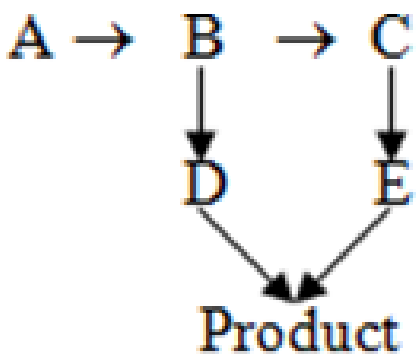


Figure 1

$$\frac{1}{\text{Cell mass}} \times \frac{d\,(\text{cell mass})}{dt} < \text{zero}$$

Type III, Secondary, or Non-growth Associated products

Secondary products result from biosynthetic reactions where the main product does not result from energy metabolism. Cell and metabolic activities reach maximum in the early stages of the life cycle and product formation takes place at the later stages of the life cycle. Oxidative metabolism is low at the time of maximum product formation e.g. Antibiotic fermentation and biosynthesis of Vitamins. In Non-growth associated product formation a period of negative specific growth rate occurs where the terms

This shows that the population has moved beyond the stationary phase for the latter part of the fermentation. Under such conditions dead cell lysis may provide a second nutrient source for the other cells. Fermentation processes are the prime sources of over hundred products for food, chemical and pharmaceutical industries. Various fermentation products are as follows:

Antibiotics: Fermentation industry once dominated by alcohol and solvent making now derives its primary income from antibiotics like *Penicillin, Streptomycin, Vancomycin, Neomycin, Chloramphenicol, and Erythromycin* etc.

Steroids: Transformation of steroids i.e. induction of hydroxyl in 11β *11*β and 16β position, dehydrogenation of 1,2 position and hydrolysis of esters of the 3 hydroxyl group can be achieved by fermentation process. Key steroids in microbial transformation process are Cortisone, Hydrocortisone, and Prednisolone etc.

Enzymes: Many enzymes can be produced by fermentation process. These find application in production of food, chemical and medicines e.g. amylases, pectinases, proteases, cellulases, catalases, invertases, lipases, streptokinases glucose oxidases and collagenases.

Organic Acids: Organic acids find their use in food, chemical and medicines as an acidulant, sequestrate, plasticizer, flavouring and reducing agents. Commonly used organic acids are citric acid, lactic acid, gluconic acid and itaconic acid. Amino acids like lysine is used as food supplement glutamic acid as a flavouring agent and are ascorbic acid as a reducing agent.

Vitamins and Growth factors: Fermentations have been used to produce growth factors for many years. These are used in pharmaceuticals and as food and feed supplement e.g. Riboflavin, vitamin B_{12}. Gibberellin is used in germinating barley and ripening fruits, Xanthophylls produced by algal cultures is added to chicken feed to give color to egg yolks and chicken meat. Torula yeast is added in animal feed as a source of B vitamins is derived by fermentation of waste liquors from paper industry.

Solvents: Many solvents like alcohol, acetone and butanol are prepared by fermentation process. However some of them now are produced by synthetic process.

Polymers: Dextran is the only polymer that is being produced on large scale by fermentation process. It is used as a blood extender and blood thickener.

Miscellaneous: Sorbose (an intermediate in ascorbic acid manufacture), fructose (a liquid sweetener), dihydroxyacetone (a sun-tanning agent) and phenylacetylcarbinol (an intermediate in L-ephedrine synthesis) are a few miscellaneous compounds produced by fermentation process. Spores of Bacillus thuringiensis used as an insecticide for chickens, is also prepared by fermentation process.

Probiotic

A bacterial supplement of a single or a mixed culture of selected non-pathogenic bacterial strains is termed as Probiotic. The term 'Probiotic' was firstly coined by Parker (1974) and originated from two Greek words 'pro' and 'bios' which means 'for life'. Probiotic generally includes bacteria, cyanobacteria, fungi etc. They may be called as normal micro biota or "Effective micro biota". Probiotic, Probiotic, Probiotic bacteria, beneficial bacteria, or friendly bacteria are the synonymously used for probiotic bacteria. According to some recent publications, the mechanisms of action of probiotic bacteria have several aspects: 1) they competitively exclude the pathogenic bacteria or produce substances that inhibit the growth of pathogenic bacteria (e.g. bacitracins and polymyxins produced by *Bacillus spp*.) 2) provide the essential nutrients to enhance the nutrition of the cultured organisms 3) they may directly uptake the or decompose the organic matter or toxic materials in water improving the quality of water in the mediums or in the treatment of water. Beneficial effects of the Probiotic may be mediated by: 1) Neutralization of the toxins 2) suppression of the viable count 3) production of antibacterial substances 4) competition of adhesion sites 5) Alternation of microbial metabolism 6) Stimulation of immunity of the host 7) Accelerate the sediment decomposition by producing organic acids in water treatment 8) production of hydrogen peroxide 9) production of enzymes.

a. Types of Probiotic

i. Non-viable Probiotic –these are dead.

ii. Freeze-dried Probiotic –these will die rapidly upon leaving refrigeration.

iii. Fermentation Probiotic –these are produced through fermentation.

iv. Viable Probiotic–these live with guaranteed shelf life. Guaranteed number of organisms has a protocol for counting and to be very stable and efficacious. Produce many benefits.

b. Bioreactor or fermentor

Bioreactor is a device in which biochemical transformations take place. It is here a less expensive material is converted into a more valuable product or

service is rendered. Even though the term is new but the concept is old. The terms like bioreactors, microbial reactors, fermentors, and biochemical reactors all have the same meaning. Until about four decades ago, fermentation had been practiced as an art with a little engineering input, but with the realization of the potential of this process, the need of its instrumentation and control was felt. With proper instrumentation and control of a bioreactor it is possible to increase the conversion yield and the productivity of a biological product manifolds. The first step in understanding, controlling and optimizing a process is the precise, accurate and timely monitoring of important parameters. The state of the art in automated monitoring is very advanced in mature industrial sectors but even today it is adapting sensors developed for other applications or designing new sensors to satisfy its needs.

c. Measurement system

From system's point of view, a measurement is achieved through the use of a meter or sensor expanding the human senses ability to detect measure and quantify. To control a fermentation process we need to know 1) state of the process within a small time increment i.e. continuous monitoring of the state variables 2) The microorganism's response to any set of measurable environmental conditions i.e. a control model for fermentation. Before describing the various sensors available for a bioreactor, it needs to emphasize the requirements of an ideal sensor. The requirements and characteristics must be met within reasonable limits; it may vary from one case to another.

These are:

i. Reliability-Long term reliability is of great importance and a time of about 2000-3000 hours continuous operation should be attainable for most instruments

ii. Repeatability or Reproducibility -Measurements made under standard conditions should be repeatable from day to day and from laboratory to laboratory

iii. Accuracy- Accuracy is the measure of how close the empirical measurement is to the true value or its conformity to an accepted standard value

iv. Rough and Tough- Sensor should be rugged and repeatedly withstand the conditions of steam sterilization, variety of chemicals besides acidity, basicity, salinity, and water etc.

All the fermentation sensors can be categorized as 1) Physical environmental sensors-to measure the physical process variables and 2) Chemical environmental sensors-to measure the chemical process variables.

d. Methods of Measurement of Process variables

Physical process variables

a. Temperature: It is an important parameter in the biochemical process. This is not true only further reaction itself, but also for auxillary operations such as sterilization and downstream processing. Temperature is usually measured with the help of Resistance thermometers, Thermocouples and liquid expansion thermometers. Risk of contamination is minimal with all these methods. Resistance thermometry prevails because of its accuracy and reliability. Sensors usually are the encased platinum wires. The use of thermocouples is less frequent. Electric measuring signals from both the resistance thermometers and thermocouples can be transferred to control boards. This is not possible with liquid expansion thermometers like mercury or ethanol in glass, which occasionally are employed for direct on the spot measurements. Another method of on the spot temperature indicator is by means of thermo colors that change their colors at certain temperature. They can be applied in the form of thermo foils attached at critical spots. Some details of the devices used to control the temperature are as follows:

b. Mercury in glass thermometers: They may be used in small bench fermentors and are very fragile in nature. That is why their use is restricted. They usually are used enclosed in a pocket which protects the glass. They are used only as an indicator.

c. Bimetallic thermometer: It consists of bimetallic coil surrounded by protective tubing. The coil Winds or unwinds with changes in temperature causing movement of fixed pointer onto it. They are more expansive and less accurate.

d. Pressure bulb thermometer: It is basically a pressure gauge connected by a small bore tubing which may be up to 60m in length to the detecting bulb. The whole system is gas tight and filled with an appropriate gas or liquid under pressure. The movement of the free end of the receiving element can be used to operate a pen on a chart recorder or an electrical or pneumatic control. (Figure 2)

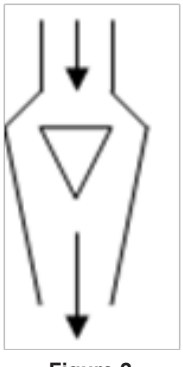


Figure 2

e. Thermocouples: In 1821 Seebak discovered that if a circuit consisting of wires of two dissimilar metals had the junction of the wires maintained at different temperatures, a current flowed through the circuit. This current produced can be measured on a calibrated

instrument or recorder and is a measure of point temperature. At a point therefore by holding the temperature constant at all junctions except one, which is a given circuit, it is possible to measure temperature with reference to the old junction temperature. Thermocouples have limited use because they are normally operated at ambient temperatures.

f. **Electrical resistance thermometers:** Electrical resistances of metals changes with temperature variations. The bulb of electrical thermometers contains a resistance element, a mica framework (for accurate measurement) or a ceramic framework, around which the sensing element is wound. Platinum wires of 100Ω resistance are normally used. The wires are then connected to the measuring element. Reading is normally obtained by a wheat-Stone bridge circuit and is the measure of the average temperature of the sensing element. The electrical resistance thermometers are very accurate, more sensitive to small temperature changes and are very fast.

g. **Thermistors:** These are semiconductors made from specific mixtures of pure oxides of iron, nickel and other metals. Their main characteristic is a large change in resistance as a function of absolute temperature. Temperature reading is obtained with a wheat-Stone bridge. They are relatively cheap and stable.

h. **Pressure:** It is measured by means of conventional pressure gauge. Since the manometer is not in the direct contact with the fermenter contents therefore no sterility problem arises. Often measurement of pressure is not included in the standard equipment, though it may yield valuable information especially with laboratory glass vessels. Here any clogging of exhaust pipe may cause a buildup of a pressure head, and thereby apart from the danger of cracking the glass. Other parameters, such as solubility of gases will be affected. In fermentors containing cultures, which tend to form wall growth, deposits of microbial mass on membranes may lead to errors in the monitoring of the pressure.

i. **Flow Rate:** Gas flow rate is important in aerobic fermentations. Likewise the rate of gas production is of interest for cultures producing biogas. Liquid flow rates must be known for continuous and fed batch processes, where the rate of nutrient feed is an essential variable for efficient operation of the process by means of mass balancing control. Furthermore, knowing the liquid flow rate is necessary to control the addition of corrective liquid feed streams, such as amount of base or acid consumed for pH control or the amount of antifoam input. Flow rates are mainly measured by the following devices: 1) Floating body flow meter 2) Differential pressure flow meter 3) Rotating flow meter 4) Electromagnetic flow meter Floating Body Flow Meter Impeller Speed For stirred tank fermenter, impeller speed is an important operating variable, which is very often kept constant. It is usually measured by monitoring the number of revolutions per unit of time. Outside the aseptic area, the impeller speed is measured with the help of a device called Tachometer. (Figure 2)

j. **Power Input:** The power consumption of agitators depend on stirrer speed and physical properties of the stirred fluid especially on its viscosity, which may change drastically during batch fermentations e.g. in some processes for the production of antibiotics like penicillin viscosity changes take place. In large-scale fermentors, the consumption of electric energy as determined by a Wattmeter, yields useful information on the input of agitating power when friction losses in the stuffing box, seals, and motor are accounted for. A direct measurement of agitation power is possible by using Torsion dynamometer or Strain gauges. The latter method is an accurate method.

k. **Foam:** It is a nuisance occurring in most fermentation broths. It may be caused by surface-active metabolites (proteins, polysaccharides etc.), components of the medium, or by cells. Two types of foams have been distinguished 1) Soft foam 2) Hard foam Soft foam is unstable while hard foam is stable. Foam of either form must be suppressed in order to prevent the contamination, clogging of the exhaust system including its measuring devices and loss of culture broth. Foam destruction can be achieved by mechanical or by chemical methods (antifoaming agents). Often both the methods are used in combination. Foam control necessitates its detection. This can be achieved by employing sensors mounted inside the fermenter above the liquid level. Examples of the various probes used are: Electric conducting probe, Capacitance probe and Heat conducting probe.

l. **Volume:** Information on liquid volume is essential in liquid flow control in the filling of the vessel with medium or in continuous culture feed in the continuous culture, which ultimately affects the metabolic activity of the inoculum culture? There are two systems generally used for the on-line volume determinations: 1) liquid level sensors and 2) weight or mass measurement devices. Capacitance probes measures the change in capacitance when liquid level changes in small-scale fermentors. In large-scale fermentors ΔP measurement method is generally used, where a flush mounted diaphragm pressure cell is located in the base of the vessel for liquid height and hence the volume measurement.

m. **Weight/Mass:** Scales of various types determine weight or mass of the liquid. In this method the vessel is suspended on a scale and the combined weight of the vessel and strain type gauges electronically measure liquid. This method cannot be applied to the fixed existing installations.

Chemical process variables

a. **Exhaust gas analysis:** The concentration of carbon dioxide in the exhaust gas from cell reaction is indicative of the respiratory activity and fermentative activity of the inoculum culture and hence is one of the most useful and widely applied measurement methods in the monitoring and controlling a cell bioreactor. Using an Infrared spectrophotometer, gas chromatography and mass spectrometer most commonly controls carbon dioxide content in a bioreactor. Gas stream oxygen partial pressure is usually measured using a paramagnetic analyzer. Care should be taken in both the cases that water vapors should be eliminated from the exhaust before feeding them into the analyzer. The paramagnetic analyzers are quite sensitive to small changes in total atmospheric pressure so they require simultaneous monitoring of barometric pressure for compensation in oxygen analysis. Apart from water vapors the samples should also be free from dust particles, aerosols and oil droplets.

b. **Dissolved gases and volatiles:** Dissolved oxygen and carbon dioxide are both important variables in fermentations. These are normally determined by 1) Electrochemical methods 2) Fluorescence quenching and 3) Mass Spectrometry Electrochemical Methods- Electrochemical determination of oxygen and carbon dioxide in fermentation media is performed by means of special sterilizable electrode. Analysis by this electrode is based on detecting the amount of oxygen diffusing from the liquid membrane into an Amperometric or Polarographic measuring cell. Amperometric principles are most frequently used. In Polarographic type oxygen electrode a constant voltage is applied between cathode and anode. At cathode the oxygen that has diffused into the cell is reduced to hydroxyl ion as shown below:

Cathode: - $O_2 + 2H_2O + 4\ \bar{e} \rightarrow 4\ OH^-$

Anode: $4\ Ag + 4\ Cl^- \rightarrow 4\ AgCl + 4\ \bar{e}$

The response of the probe is proportional to the oxygen activity in liquid. Since at equilibrium i.e. for a saturated liquid the activity of a solute is directly related to its partial pressure (fugacity), the readings of electrochemical process are commonly given as percent partial pressure or saturation.

In Potentiometer probe same principle of oxygen diffusivity is used and this diffused oxygen is reduced at cathode surface according to the same above equation:

Pt

$\frac{1}{2}\ O_2 + H_2O + 2\ \bar{e} \rightarrow 2OH^-$

The reaction at anode in galvanic electrode is as follows:

$$Pb \rightarrow Pb^{2+} + 2\ \bar{e}$$

This reaction competes with the cell from which a small amount of current is drawn to provide a voltage measurement, which in turn is correlated to oxygen flux reaching the cathode surface. Fluorescence Quenching- For medical investigations so called Optodes has been developed for oxygen and carbon dioxide determinations. In this, the sensitive element is the membrane into which a fluorescence indicator (Pyrene butyric acid or β- methyl belliferon purine) has been incorporated. This membrane is brought in contact with the broth. The fluorescence quenching in indicator is indicative of the presence of oxygen or carbon dioxide. This method does not consume oxygen or carbon dioxide.

c. **pH:** pH value is an important indicator of the state of the state of biochemical process. The automatic addition of alkali or acid to fermentation broth can be achieved by techniques already in use in other chemical industries but special electrodes have been developed for use in fermentation industry. The half-cell of the glass electrode was composed of Ag/AgCl saturated with KCl. The solid KCl increases the mechanical resistance of the glass particles of KCl on the glass surface during heat sterilization and cooling of the electrode. Other half of the electrode is composed of the same material as the glass electrode, asbestos or porcelain cylinder being used as the junction material. To ensure good insulation, both the glass and reference electrode were mounted in Teflon gaskets and silicone rubber washer were fixed. The internal resistance of the electrode is 300-500meg Ohm. Steel sleeves provided with several holes to allow free passage of broth protect both of these electrodes.

d. **Redox potential:** Another method obtained with electrochemical method is the Redox potential measurement method. Every redox system consists of two components, one is oxidized by electron donation and the other is reduced by electron acceptance. In such a system, an electrochemical potential can be measured by means of an unprotected electrode consisting of a noble metal (Au/Pt), the composition of which is chosen on the basis of relation of donor to the acceptor. In a fermentation system/culture, a great number of redox systems are present simultaneously. Accordingly an exact interpretation of signals from the redox potential measurements cannot be given. It is for that reason that some scientists suggested to rather name this potential as Platinum-electrode potential instead of redox. The competing donors i.e. oxygen and glucose that are present in a fermentation system may serve as a typical example. In spite of the difficulty of interpreting the results, measurements of redox potentials permit an important insight into the course of fermentations. Sterilizable Platinum electrodes are commercially available. They either contains built in reference electrode (Ag/AgCl) similar to pH electrode or they are used in combination with pH measurement making use of the same reference electrode. The amplifier for redox measurements is of the same type as in conventional pH meters. As in the latter case, the built in electrode is sterilized together with the fermenter. Since no membrane is required so any

special sterilization problem exists in these electrodes. The signal of the redox meter is influenced by pH. This can be tolerated because fermentations are usually run at constant pH.

e. **Enzymatic analysis of substrate:** Enzymatic analysis allows very specific determination of many organic compounds. This group of methods takes advantage of the ability of enzymes to react selectively with well-defined compounds or rather chemical structures; organic compounds present in fermentation media (substrates and metabolites) can be analyzed. Usually this is performed offline with samples taken from fermentors but methods for online enzymatic analysis have also been developed. The procedure consists of determining the conversion of the enzyme-catalyzed reaction with the respective substrate for analyzing one of the products either calorimetrically or electrochemically. In applying online enzymatic analyses to sterile fermentations, special difficulties arise because conventional sterilization techniques apply steam at 120˚C that will destroy the enzymes. One solution to this is to employ dialysis through which a recycled sample stream of broth is conducted. Components diffusing through the dialysis membrane can then be monitored continuously by means of enzymatic analysis. Such systems have been developed for measuring glucose, saccharine and lactose.

f. **Ion specific electrodes:** Not only carbon source and other organic compounds but also inorganic salts (N, P, S, K, Mg, Ca, Na, and Fe) are essential constituents of fermentation broths. Ion specific electrodes have been proposed for a number of these ions. Some electrodes of this type are commercially available for offline measurements. There is little known about the online measurement using ion selective electrodes. Ion selective electrodes are Infact potentiometer electrodes applying different principles e.g. for measurement of Na+ glass electrodes with glass membrane especially sensitive to Na+ are employed. In some electrodes organic membranes are employed while in others enzymes may be incorporated. For these types of electrodes sterilization may be the problem.

How Temperature and pH Affect the Growth of Microorganisms

Effect of temperature on the growth of microorganisms

Bioprocesses of microorganisms are heavily affected by the temperature. The cell temperature must become equal to the culture temperature. Temperature affects the rate of cell reactions, the nature of metabolism, the nutritional requirements and biomass composition. The temperature coefficient of growth rate is denoted by Q10 value (It is defined as increase in growth rate per 10°C rise in temperature e.g. If Q10 is equal to 2 that means there is two fold increase in growth rate per 10 °C rise in temperature. Growth rate approaches zero at 10 to 25 °C below the optimum temperature. Chemical reaction rates are related to temperature by Arrhenius equation:

$$K = A\, e^{-E/RT}$$

Where K = reaction rate; A= Arrhenius constant; R = Gas constant; T = Absolute temperature; E = Activation energy or temperature characteristics.

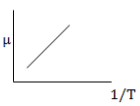
Taking log on both sides we have:

$$Log\ \mu$$

$$1 / T$$

Plot of Log K against 1/T should be a straight line with slope of E/2.3 RT. If we substitute specific growth rate 'μ' for reaction rate K in the above equation then converting it to straight line equation and plotting log μ against 1/T, keeping the value of E constant we find a straight line with slope of E/2.3RT. The Q10 value also varies inversely as the temperature varies from normal as

$$Q_{10} = E \,.\, 10 / 2.3RT\ (T+ 10)$$



The activation energy is a valuable constant as it can be used to predict the effect of temperature on growth rate over the normal temperature range. Changing in the activation energy indicates that differences in rate controlling reactions or in the metabolic regulations can occur. Temperature range for growth of individual bacteria extends over about 35°C. Extreme psychrophiles grow between -5 to 30°C and extreme thermophiles 55 to 90°C. Decrease in growth rate at high temperature is due to disruption of metabolic regulations or death of cells by protein denaturation. When death of cells occur the growth rate of the viable biomass (X) is given by:

dX/dt = (μ -K) X where μ = specific growth rate; K = specific death rate and X = biomass

Death rate becomes dominant if at high temperature activation energy for death exceeds that for growth. Increase in temperature causes breakdown of protein structure so the affinity for substrate and enzyme regulators will be affected. Thermophiles possess proteins with exceptional heat resistance. Temperature also affects the nutrient requirements, lowering of the growth temperature causes small increase in the growth yield from carbon and energy source. The pathways of metabolism of carbon and energy source can be temperature sensitive e.g. *Lactobacillus brevis* ferments glucose by heterolactic pathway at 24°C but at 32oC requires fructose as hydrogen acceptor for glucose fermentation. Growth factor requirements also change with temperature e.g. *Yersinia pestis* requires different amino acids and vitamins at growth from 37 °C to that at

28°C. Temperature affects the product formation e.g. over production of riboflavin by Ashbya gossypii requires growth of the microorganism at 28°C than at its normal temperature because growth at low temperature causes breakdown of normal regulation of synthesis of enzyme system which produces the riboflavin. Similarly the optimum temperature of production of Penicillin is lower than that of normal growth temperature. Temperature affects the microbial composition as RNA content of bacteria or yeasts increase several folds on decreasing the temperature. Yeast lipids increase their unsaturated fatty acids when temperature is lowered. Antigenic composition of bacteria varies both qualitatively as well as quantitatively with temperature e.g. virulent Yersinia pestis is produced at 37°C but not at 25°C.

Mechanism of temperature effect

The effect of temperature can be explained as: 1) Dependence of structure of cell components on temperature, 2) Activation energy required for the reactions to occur inside the cell which in other term affects the regulatory mechanisms of the cell, cell composition and permeability functions.

Effect of pH on the growth of microorganisms

The influence of [H+] on biological activities is related to either hydrogen ion concentration or hydrogen ion activity (ah). These two parameters are proportional as:

ah = f [H+] where f is the activity coefficient which may vary with the ionic strength and other factors. The glass electrodes respond to hydrogen ion activity so that strictly pH = -log (ah) and [H+] can be substituted for hydrogen ion activity. It can only be possible when activity coefficient is one. In dilute media solutions f approximately is one. But this may be far from true when media are strong salt solutions. As far as cell properties are concerned hydrogen ion activity is more meaningful parameter so that it is appropriate to express the effect of hydrogen ion concentration in terms of pH. Plasma membrane is not freely permeable to hydrogen ions or OH- ions. So that intracellular and extracellular hydrogen ion concentrations do not necessarily equilibrate and a gradient of hydrogen ion across the plasma membrane is established. According to chemiosmotic theory this gradient of hydrogen ions together with membrane electric potential makes a proton motive force that derives the membrane reactions.

Lactic Acid

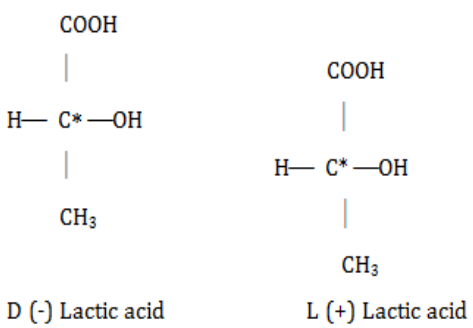
Scheele (1789) first isolated lactic acid from sour milk. The studies on the physical and chemical properties have shown that the compound occurs in two isomeric forms and as a mixture of the two isomeric forms. Lactic acid is also produced in muscles. Many microorganisms produce lactic acid by the fermentation of sugars. The structural formula of Lactic acid is CH_3 -CH (OH)–COOH.

Technological development

Lactic acid was the first organic acid to be manufactured industrially by the fermentation process. The first commercial production of lactic acid in USA by microbiological process took place in 1881 as its calcium salt Calcium lactate. The plant for its manufacture was built in Littleton, Massachusetts, but little is known about its process. The Clinton processing Company, Clinton Iowa is the only manufacturer using fermentation process for the production of lactic acid in United States.

i. **Isomers:** Lactic acid of commerce is the L (+) isomer, D (-) isomer or any possible mixture of the two. The entire range of isomers has been found. The mixture of the two forms of isomers is called as DL mixture or racemic mixture. DL mixture is optically inactive form. Microorganisms differ in their ability to produce either D (-) lactic acid or L (+) lactic acid or racemic mixture and the particular acid formed are the characteristic of individual microorganism. From industrial standpoint the lactic acid recovered from fermentation broth usually is the racemic mixture because fermenting microorganisms or contaminants like Lactobacillus plantarum produce an enzyme known as Racemase that converts either of the optically active isomer to optically inactive racemic mixture. Some trace impurities in the medium also have been reported to bring about racemization. The Racemase are known to be lactic dehydrogenises that maintain equilibrium between lactic acid isomers and pyruvic acid. When both dehydrogenase enzymes are present, racemization occurs.

ii. **Microorganisms:** Many types of microorganisms have been isolated that accumulate lactic acid or lactates in the culture solutions e.g. Lactic acid bacteria, algae, molds, yeasts and phycomycetous fungi. Apart from many Lactic acid bacteria, mold Rhizopus oryzae has been found to produce lactic acid comparable to homofermentative lactic acid bacteria from glucose. Lactic acid bacteria fall under two main groups: (1) Homofermentative Lactic acid Bacteria (2) Heterofermentative Lactic acid Bacteria



iii. **Homofermentative lactic acid bacteria:** These bacteria produce lactic acid as the major or sole product of glucose fermentation. The homofermentative

pattern is observed when glucose is metabolized but not necessarily when pentoses are metabolized, for some homolactics produces acetic acid and lactic acid when utilizing pentoses. Also, the homofermentative character of homolactics may be shifted for some strains by altering cultural conditions such as glucose concentration, pH and nutrient limitation. The homolactics are able to extract about two times as much energy from a given quantity of glucose as are the heterolactics. They are less important in producing flavor and aroma components e.g. acetaldehyde and diacetyls in food products. They possess the enzymes aldoses and hexose isomerase but lack phosphoketolase. They use EMP pathway to produce two molecules of lactate per glucose molecule. Examples of homofermentative lactic acid bacteria are: All members of genera *Streptococcus, Pediococcus* and some genera of *Lactobacilli* like *L. delbrueckii* Homofermentative lactic acid bacteria are very important for the production of Lactic acid industrially.

iv. **Heterofermentative lactic acid bacteria:** They produce some lactic acid, but at the same time, they also produce carbon dioxide, ethanol, and acetic acid and trace amounts of a few other products. These organisms are of little use for industrial lactic acid fermentations because too much of the substrate carbon is directed towards products other than lactic acid. The end product differences between homo and Heterofermentative lactics when glucose is attacked are a result of basic genetic and physiological differences. The heterolactics have phosphoketolase pathway but do not possess aldolase and hexose isomerase. Instead of EMP pathway such organisms use Hexose monophosphate shunt as energy pathways. Heterofermentative bacteria are very important for the production of flavor and aroma components like diacetyls in food products. Examples of Heterolactics are: All species of genus Leuconostoc, and some species of Lactobacilli.

Microorganisms for Commercial Production of Lactic acid

The microorganisms used for commercial production of lactic acid depends upon the raw material to be fermented, but the most common bacterium used for this purpose is *Lactobacillus delbrueckii*; it is employed in fermentations utilizing corn dextrose medium. Although increasing use is being made of a flat sour *Bacillus coagulans* yet *Lactobacillus bulgaricus* is used for the production of lactic acid from whey because it utilizes lactose as a carbon source. *L. pentosus (L.plantarum)* is recommended for use in spent sulfite liquor, as it is able to utilize pentoses. L. brevis is used when medium contains hydrolyzed corncobs, cotton seed hulls etc. Other homofermentative species of potential industrial importance are *L.casei, L.leichmannii*, and *Streptococcus lactis*. These are facultative anaerobes and can withstand some oxygen. *Streptococcus lactis* is particularly useful under such conditions.

Medium

The fermentation solutions usually contain hydrolyzed starches or dextrose syrup, although D-glucose, maltose, lactose or sucrose can also be fermented. It is advantageous to start with a relatively simple medium or mash in order to facilitate recovery of the product. Crude carbon sources are generally avoided because the impurities interfere with the recovery and purification procedure. The sugar concentration in the medium should not be more than 12-15% because Calcium lactate produced at high sugar concentration tends to crystallize from the medium late in the fermentation, thereby slowing the fermentation process. Nitrogen sources are added in small amounts and are usually inorganic in nature e.g. Ammonium phosphate. This is because impurities in the nitrogen sources might interfere in the recovery and purification procedure. Calcium carbonate (10%) is added to neutralize the lactic acid produced because lactic acid bacteria cannot tolerate high acid concentration. Lactic acid bacteria have complex requirements of B-vitamins. This ordinarily is met by enrichment of the culture medium with crude vegetable materials. Malt sprouts are commonly used vegetable materials, but if they have been overheated in drying, they lose some of their value as a nutrient for *Lactobacilli*.

Production of Lactic Acid

Today much of the lactic acid is produced by the hydrolysis of lactonitrile, a byproduct of another process. Only a few companies in the world are producing lactic acid by fermentative process.

Equipment

Lactic acid is very corrosive to metals therefore wooden fermentors are used in most of the plants. These fermentors may be uncovered or covered with loosely fitting wooden lids. They are to be steamed empty before charging. Fermentation solutions are pasteurized or sterilized by passing it through a steam jacketed heat exchanger. Contamination of culture solutions sometimes by *thermophilic Clostridia* results in the production of some butanol and butyric acid. Such contaminated lactic acid could only be sold to leather tanners for delining of hides. A very pure lactic acid is required for manufacturing of plastic. Such grade of lactic acid is called Plastic grade lactic acid.

Inoculum

Cultures of *L. delbrueckii* are transferred from test tubes through successively larger culture vessels, held at 45-55 °C. Each stage of the culture build up requires 16-18hours and slight excess of Calcium carbonate is required at each stage. Inoculum volume should be 5% of the volume of fermentation solution.

pH

An excess of Calcium carbonate keeps the pH in the range of 5.5-6.5. The pH necessary varies with the composition of culture solution but it is controlled by continuous

neutralization with the slurry of Calcium hydroxide between 6.3-6.5. Fermentations utilizing grain may resist increase in pH with the buffering capacity of mash.

Aeration and agitation

The medium is not aerated but agitation is done to keep the Calcium carbonate in suspension. Fermentation Time and Fermentation Temperature: The fermentation temperature is adjusted to 45-50 °C and varies the type of organism used. Same is the case with fermentation time. It is usually completed in six days or less depending on the time required by the organism to deplete the sugar in the medium. Fermentation time is usually 5-10 days. It is important that residual sugars be reduced to 0.1% or less during the fermentation because residual sugars make recovery of better quality lactic acid difficult.

Yield

Commercial yields are 93-95% by weight of glucose supplied. Recovery yields vary with the various recovery processes and product grades.

Product Recovery and Grades

Technical grade lactic acid

Calcium from the fermentation solution is precipitated as Calcium Sulfate, filtered, and filtrate is evaporated to 35-40% lactic acid. Now more Calcium sulfate is precipitated, filtered, and filtrate is evaporated to 44-55% total acidity. It is then crystallized.

Food grade lactic acid

It is a pale yellow, straw colored solution of about 50% total acidity. Calcium in this case is precipitated as Calcium sulfate, precipitates are washed, washed water is combined with the filtrate, filtrate is bleached with activated carbon and it is then subjected to evaporation firstly to 25% solids, again bleached and then secondly evaporated to 50% solids, bleached finally to produce an off colored product. Impurities like Iron and Copper metals are removed by adding Potassium ferrocyanide in the filtrate of first filtration.

Plastic grade lactic acid

It is a colorless product. Product is recovered by etherification with methanol after concentration or by solvent extraction with isopropyl ether followed by re-extraction of isopropyl ether with water.

Pharmaceutical grade lactic acid (Lactic Acid USP)

It is a colorless product with 85% total acidity and 76-78% concentration. It contains 2-3.5% volatile acids 0.5-1.0% ash content.

Uses of Lactic Acid

In the foods, lactic acid is added to acidify jams, jellies, confectionary, sherbets, soft drinks, extracts and other products. It is added to brines for pickles and olives and to fish to aid preservation. Its addition makes milk more digestible to infants. Calcium lactate is an ingredient of some baking powders. In tannery, it is used for washing of hides. In textile industry it is employed for fibre washing. Lactic acid is used in the preparation of medicines. It is also used as a laboratory reagent and a research tool.

Citric Acid

Scheele first isolated Citric acid in 1784 from Lemon juice by crystallization process. Members of citrus family of fruits especially are rich in this organic acid, but citric acid is also found as a natural constituent of a variety of fruits. Citric acid extracted from fruits is designated as Natural Citric acid in contrast to the Citric acid produced by Microbial Fermentation process. Citric acid can also be prepared synthetically but no equivalent synthetic process has been invented to the microbial fermentation.

Microorganisms

Many microorganisms like molds (*Aspergillus niger, A. awamori, Penicillium janthinallum, Trichoderma viridae, Mucor piriformis*, etc.), yeasts (*Candida lipolytica, C.tropicalis, C.citrica, Hansenula, Rodotorula, Pichia, Torulopsis* etc.) and bacteria (*Bacillus licheniformis, Bacillus subtilis, Brevibacterium flavus, Corynebacterium species*) have the capability to produce citric acid. Commercially spores of Aspergillus niger are employed to produce citric acid.

Methods of fermentation

Fermentation of Citric acid is carried out by any of the following methods 1) Stationary or Surface culture, 2) Submerged Culture, 3) Solid State Culture, 4) Continuous Culture, 5) Multistage Culture process, 6) Semi-Continuous Culture process.

In stationary or surface culture, sterile nutrient medium with sugar is added into stainless steel or high-grade aluminum trays sterilized with formaldehyde or sulfur dioxide. Spores of *Aspergillus niger* are inoculated and incubated at 28-30 °C for 8-12 days. Submerged process consists of two phases i.e. growth phase and productive phase. In Growth phase medium is inoculated with spores *of A. niger*, after 3-4 days mycelium is separated from solution and added to the fermentation medium and fermentation is allowed to occur for 3-4 days. During the period production of the Citric acid takes place. This phase now is called as Production phase. In Solid state Culture as described by Calm (1935), fermentation medium is impregnated in porous solid materials like sugarcane baggase, potato or beet pulp, or pineapple pulp in an appropriate ratio, sterilized and then inoculated with a suspension of fungal spores and incubated at 25-30 °C for 6-7 days. In Continuous or multistage culture, the medium is replaced after 24 hours and that medium goes to second fermenter. It is then

aerated by gradually increasing the amount of air. In Semi-Continuous Culture, the whole medium is not replaced but a part is replaced.

Medium

Components of the medium varies with the type of the process used because it has been seen that the strains that give good result in surface culture do not perform better in submerged culture. Fungi have been seen to give much better results in simple synthetic media. A variety of carbon sources are being used these days, which include Sucrose, Citrus molasses, Cane juice, Starch from various sources, Cane or Beet molasses. The initial sugar content determines the amount of citric acid produced by *A. Niger*. Normally 15-18% sugars are added into the medium. Concentration of sugar more than 15-18% leads to greater amounts of residual sugars that make the process uneconomical. When the sugar concentration is lower than the above percentage, it leads to lesser yield of citric acid as well as accumulation of oxalic acid. Molasses contains 50-60% sucrose and is of three types: 1) Black Strap molasses- it is obtained from the last stages of crystallization of sugar. 2) Refinery molasses- it is obtained at the second stage of refining sugar. It contains 48-50% sucrose and has high ash content. 3) Invert or High-test molasses- it is partly inverted cane juice syrup from which no sugar has been extracted. Molasses is diluted to sugar concentration of 15-20% and pH adjusted to 5.5-6.5 with sulfuric acid. Molasses is added with other required nutrients and mixture is sterilized for 30 minutes. For the growth stage of *Aspergillus niger*, the organism needs major elements (C, N, P, S) and trace elements. The type of inorganic source and its concentration affects the performance of the fungus. Inorganic sources like Ammonium sulfate prolong vegetative growth. Ammonium nitrate shortens the growth phase. Ammonium nitrate in concentration greater than 0.25% accumulates more of oxalic acid whereas sodium nitrate at concentration 0.4% delays the onset of production phase and vegetative growth also increases. Phosphate also affects the citric acid yield. High phosphate concentration promotes growth and there is less acid production. Therefore phosphate concentration in the medium should be kept at 0.1%-0. 2 %. Trace elements (Fe2+, Cu2+, Zn2+, Mn2+ and Mg2+) are necessary for *A. niger*. Mg2+ is necessary for a variety of enzyme reactions in the cell. It is required for growth and production phase. Its optimum concentration in the medium should be 0.02-0.025%. Fe2+ and Zn2+ have critical role to play in the growth and production of citric acid. Both are essential for the production of citric acid in low concentration because their high concentration allows vegetative growth. Iron also has deleterious effects on the fungus but its action is counteracted by copper ions. Zn2+ at concentration 1-2 µM allows growth phase but its less concentration restricts growth. Its excess in the citric acid producing culture reverses the production phase. It also has its indirect role in the functioning of cAMP that enhances the production phase. To remove the excess of trace elements from the culture medium various methods adopted are: 1) Pretreatment of the raw materials. 2) Ion exchange resins. 3) Development of resistant varieties of fungus. The raw materials are treated with Potassium ferrocyanide to reduce the concentration of iron ions in molasses. The chemical is either added directly in the medium or the molasses is treated with its high concentration. Raw materials are also sometimes treated with chelating agents like EDTA, activated charcoal or polythene amine. Chemicals of quaternary ammonium compounds category like Diiosbutyl phenoxy ethyl dimethyl benzyl ammonium chloride and Triton-B are also used.

pH

Fungus can tolerate high concentration of acid therefore calcium carbonate is not added in the medium. The initial required pH of the medium depends upon the carbon source used as follows: Glucose or clarified molasses pH = 3.0, Crude molasses pH = 5-6, Decationized molasses pH = 1.4-2.8, Sucrose pH = 2.0-3.0. pH is adjusted with HCl, H_2SO_4 or NaOH.

Additive and stimulants

Methanol is usually used to increase the yield at a concentration of 3-4%. At this level it retards growth, delays sporulation and increases citric acid yield. This is added before inoculation. It has some role in the conditioning the mycelium without impairing their metabolism. It is thought that it increases the tolerance of the fungus towards the harmful effects of the trace elements. Other additives like hydrogen peroxide, Methylene blue, Naphthaquinone, aromatic amides, esters of dichloroacetic acid, sodium sulfite, crysyllic acid, glycerol, vegetable oils (corn oil, almond oil and peanut oil) and cAMP are added as stimulant to increase the yield.

Antifoaming agents

Antifoaming agents like Octadecanol (0.75% solution) or Antifoam AE (silicone oil) are added in the medium. To control contamination by bacteria Pentachlorophenol ate, formic acid, tetracycline's, and 3-furaldehyde semicarbazone are added in the medium.

Biochemistry of Citric Acid Formation

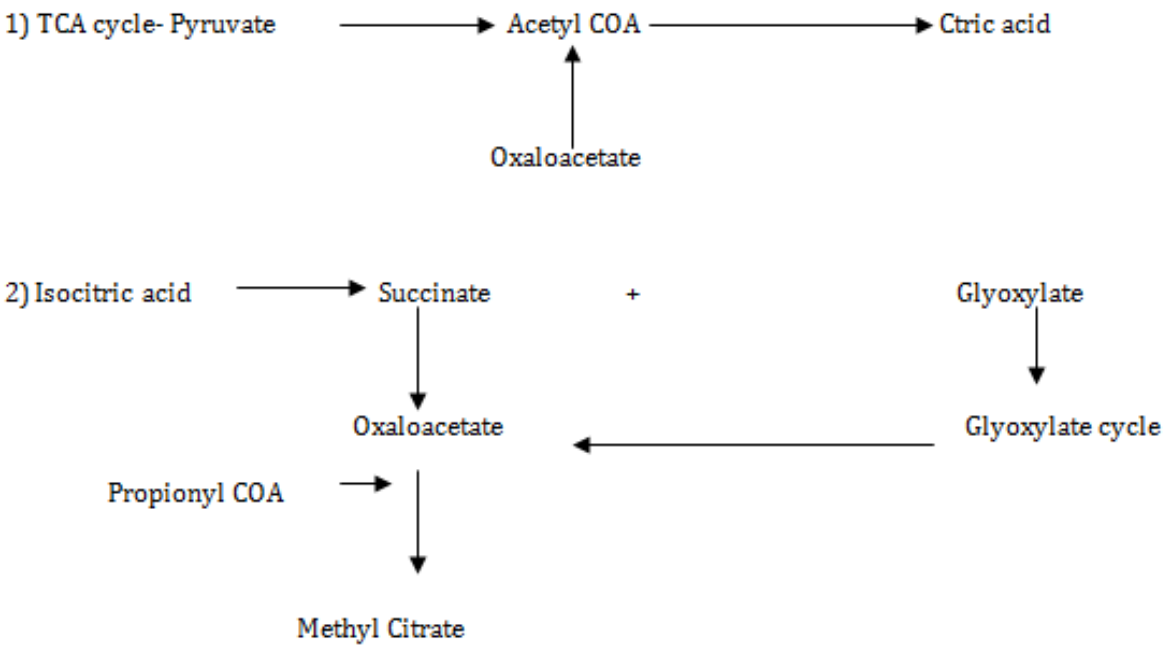
Citric acid is formed via following pathways: (Flow Chart 1)

Citric acid accumulates in culture solutions of pH 1.8-2.0. In fungi different metabolic pathways are involved in the production of citric acid but 78% of the citric acid is produced by the involvement of glycolysis and Tricarboxylic acid cycle. The acetyl COA derived by EMP pathway condenses with Oxaloacetate of Kreb's cycle to produce citric acid. Since it has been observed that, during accumulation of citric acid, *A.niger* demonstrates decreased activity of condensing enzyme and almost no activity of Isocitrate dehydrogenase and aconitase therefore another theory of its production came into light. In this pathway, glucose first splits up into

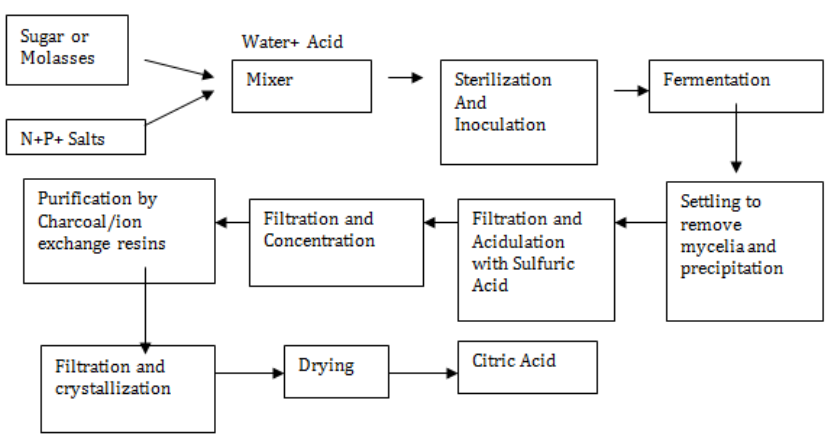
two 3-Carbon fragments followed by decarboxylation of other fragment to yield a 4-Carbon compound. The 2-C and 4-C compounds then combine to yield citric acid. In methyl citrate pathway Propionyl COA formed through the β-oxidation of n-alkanes condenses with Oxaloacetate to yield methyl citrate.

Product recovery

The crude fermented liquor is subjected to filtration to remove mycelia of fungi. Calcium citrate is precipitated from the clear liquid by adding slurry of calcium hydroxide (add hydrated lime 1 part for every 2 parts of liquor added over one hour period and temperature raised to 95°C) and heating the mixture to 80-90°C. Calcium citrate is filtered off on conventional filter and the filter cake is transferred to a tank called acidulator where it is treated with Sulfuric acid to precipitate calcium sulfate, which is then filtered. The dilute filtrate containing citric acid is purified by treatment with activated charcoal and demineralized by passing it through ion exchange resins. The purified solution is evaporated in a circulated granulator or circulating evaporator called crystallizer. Crystals are removed by centrifugation. Citric acid so prepared is subjected to recrystallization. The remaining mother liquor is added into the main stream prior to limming and decolorization (Flow Chart 2).



Flow Chart 1



Flow Chart 2

Single Cell Protein (SCP)

Definition -The dried cells of various groups of microorganisms like Bacteria, Yeasts, Molds, Higher fungi and algae that have been considered for food or feed are collectively referred to as Single Cell Protein. C.L. Wilson in Massachusetts institute of technology coined the term SCP in 1966. People have eaten certain microorganisms as a portion of their diet e.g. Top fermenting yeasts (*Saccharomyces spp*.) was recovered as a leavening agent for bread as early as 2500 B.C. Fermented milks and Cheeses produced by Lactic acid bacteria of genera *Lactobacilli and Streptococci* were consumed by early Egyptians and Greeks and reached a higher state of development during the Roman era (100-50 B.C.). Pharaohs of Egypt prized wild mushrooms as a delicacy. Romans regulated the grading and selling of mushrooms. People of Chad regions of Africa and Aztec in Mexico have eaten *Spirulina spp*. (Blue green Algae) for many generations. The purposeful cultivation of microorganisms for direct use in human food or animal feed is a fairly recent development. Considerable efforts have been expanded since World War-II to develop mass cultivation of microbial cells.

Nutritive value and use of SCP

Nutritive value of SCP varies with the microorganism used. Protein digestibility of SCP is expressed as percentage that ranges from 65-96 for various cultures. Protein efficiency ratio ranges from 0.6-2.6. Food yeasts are high in proteins and vitamins of B-12 category but may be deficient in some amino acids such as cysteine and methionine. The UN protein advisory group has major concern over the use of SCP for human beings because of the following reasons: 1) The high nucleic content of the SCP may elevate serum uric acid level, which may result in kidney stone formation or gout. 2) Certain skin reactions may occur by consuming foreign proteins 3) Possibility of carrying over of carcinogenic factors 4) possibility of gastrointestinal reactions.

Production of SCP

a. **Production of SCP by algae:** Algae can be grown photo synthetically or autotrophically in the Presence of sunlight, artificial light or heterotrophically in dark with organic carbon and energy sources.

Microorganisms

The most important algal strains used for SCP production include:

Chlorella sorokiniana, Scenedesmus acutus, S. quardicanda, S. obliqus, Spirulina maxima, S. platensis.

Growth conditions

In an open circulating system particularly sewage oxidation ponds, mixed culture of algae tend to predominate rather than a single strain. The limiting factor in the algae growth on a large scale is illumination. Carbon dioxide is the source of carbon for photosynthetic-autotrophically growing cultures. Air contains only 0.03% carbon dioxide. Accordingly, additional carbon dioxide must be supplied to the cultures. Some algae can also be grown in lakes rich in sodium carbonate. In sewage ponds, the algal growth is limited by the extent of liberation of carbon dioxide and ammonia by bacterial action. Slow and uniform liberation of carbon dioxide is necessary to provide a uniform supply of carbon source to the growing culture. Nitrogen sources suitable for algal growth include ammonium salts or nitrates. The N-sources together with phosphorous and mineral nutrients may be readily available in sewage ponds. However on other water supplies where synthetic media are used in culturing algae, these nutrients may have to be supplied. Aseptic conditions are not maintained during the large-scale growth of algae in ponds but the potential contamination by pathogenic bacteria may be given serious consideration. The key factors in a large-scale algal cultivation are agitation and flow rates. Agitation prevents sedimentation and keeps the cells in suspension while flow rate adjustments allows the detention period of algal culture to exceed the generation time. This helps in maximum population development.

Cell recovery

Cells must be recovered by concentration, dewatering and drying. The inorganic compounds like Aluminum sulfate, calcium hydroxide and cationic polymers have been investigated as flocculating agents but these do not separate from algae. Algae may auto flocculate in shallow ponds at pH 9.5 or above without flocculants. Ion exchange resins at pH 2.8-3.5 can also recover algae but this method is expensive. Spirulina maxima float on the surface in clumps when maximum growth is attained. It can be harvested by skimming at much lower cost than would be incurred by centrifugation.

SCP production by bacteria and actinomycetes

Bacteria are of interest for use in SCP production because of following reasons: 1) High growth rate (20-30 min. in bacteria, 2-3 hours in yeasts and 16 hours in algae, molds and higher fungi). 2) Bacteria can utilize a variety of carbon and energy sources, renewable sources as carbohydrates (starch, sugars and cellulose) and non-renewable sources as hydrocarbons and petrochemicals. Actinomycetes are of interest because their growth patterns and substrate utilization patterns are similar to bacteria.

Microorganisms

Methylococcus capsulatus, Methylomonas methylovora, Methylophilus methylotrophus, Pseudomonas spp.

Growth conditions

Some important considerations for selecting bacterial cultures suitable for use in SCP production are: High Specific growth rate, yield on a given substrate, pH and temperature tolerance, less aeration requirement, genetic stability and

freedom from associated bacteriophage. Criterion for using bacteria for SCP production is as follows: 1) Strains should not be pathogenic to plants, animals or human beings. 2) Strains should not have potential for mating with known pathogenic bacteria to yield pathogenic hybrids. Growth condition requirements are: 1) Carbon and Nitrogen ration in the medium should be 10:1 or less to favor high protein content in cells and to prevent accumulation of lipids or storage substances such as Poly-β-hydroxy butyrate. Nitrogen is added as anhydrous ammonia or ammonium salts and phosphates as phosphoric acid (feed grade) to avoid contamination with arsenic or fluorides found in crude industrial phosphoric acid. Minerals are added as Magnesium and Manganese in water. Other minerals are added as sulfates and hydroxides but not chlorides. pH is controlled in the range of 5-7. Temperature tolerance is important for bacteria grown on alcohol hydrocarbons. Maintenance of sterile conditions is important to avoid contamination at pH 5-7. Different carbon sources may be used for bacteria these may be carbohydrates sugar, starch, cellulose, and baggase, wheat bran, and wood, petroleum products diesel oil, gas oil, and n-hexane, proteins (Collagen, meat packing waste), methane, Alcohols (methanol) and n-alkanes.

Cell recovery

A number of problems come in way in bacterial cell recovery process because large volume of water is to be handled and bacteria have very small size in addition to that the bacterial cell densities are very close to water. Therefore they are separated by centrifugation, filtration and electrochemical coagulation methods. After separation they are spray dried.

Production of SCP by yeasts

Yeasts are probably the most widely acceptable and used microorganism for SCP production. Yeasts have the capacity to grow on a variety of substrate including waste products that contain pentose sugars. Yeasts in general have several advantages over bacteria and algae these are: 1) better public acceptance 2) lower nucleic acid content 3) easier harvesting because of size and concentration 4) high protein content 5) production of vitamins of B-12 category 6) growth in substrates of low pH.

Growth conditions

a. Raw materials used as substrates: Materials that are employed to produce SCP by yeasts

include: 1) molasses from sugar industry 2) starch after hydrolysis 3) spent sulfite liquor from paper industry 4) acid hydrolysates from wood industry 5) whey from dairy industry 6) hydrolyzed starchy foods (grains and cull potatoes, fruit wastes etc.) 7) methane 8) methanol and ethanol 9) alkanes and paraffins 10) gas oil 11) combustion gas Substrates and nitrogen concentration for yeast growth should be adjusted to provide C □ N in the range of 7: 1 to 10: 1 to favor high protein content. Concentration of carbohydrates in batch culture should range from 1-5% while in continuous culture having hydrocarbons and alcohols; lower concentrations are to be used. Ammonium salts or anhydrous Ammonia is suitable as nitrogen source. Phosphoric acid is used to adjust pH. Yeasts when grow on substrates like carbohydrates, hydrocarbons or alcohols, a lot of heat is liberated therefore heat tolerant varieties should be employed for production (e.g. Hansenula polymorpha can grow at 37-42°C) and cooling water or refrigeration facilities should be employed in the fermentation. The growth rate of yeasts under aerobic conditions depends upon the rate of mass transfer of oxygen and substrate to and across the cell surface. Yeast SCP production may or may not take place under sterile conditions. In either batch or long-term continuous yeast production one must balance the need for contamination control by maintaining sterile conditions, with the capital and operating costs of the equipment required. The temperature of fermentation is maintained at 36-37°C and pH 4.5-6.0. Yields are 45% or more of the dry yeast on the basis of the sugar fed.

Cell recovery

Yeast cells range in size from 5-8µm and have a density 1.04-1.09 gm/cm^3. They can be recovered readily from the medium by centrifugation. The cells are centrifuged in 2 stages. In the first stage it is dewatered and yeast cream is separated. This is followed by two subsequent washing centrifugations. The final washed yeast cream usually contains 15-20% solids. In SCP by *Kluyveromyces fragilis* grown on cheese whey, the entire growth medium is passed through a three-stage evaporation to concentrate the solids from 8-27% to give a feed grade product. The separated cells can either be drum or spray dried.

Ethanol as Fuel

Ethanol can be used as such or in blends with petrol as a motorcar fuel. About 10% ethanol can be used without modifications in the engine but the only disadvantage it has is that it increases the octane rating. Combustion engines can be built to run straight on ethanol or on ethanol of lower concentration (80% ethanol and 20% water). Current interest in various parts of the world centers to use a blend of ethanol called 'Gasohol', which is a blend of 90% unleaded petrol and 10% ethanol. Anhydrous ethanol is preferred for this application but 96.5% ethanol can be used if mutually miscible solvent such as isopropyl alcohol is added.

Raw materials

Forty-five kilograms of fermentable sugars (glucose) is assumed to yield 18-23 kg or 23-28 litres of ethanol. The yield is same for starchy raw materials i.e. between 40-50% based on dry weight of carbohydrates. Complete hydrolysis of starch yields about 50 kg of glucose but conversion is never complete, therefore with 90% conversion the yield is 40-50%. For cellulosic raw materials yield is less because α-cellulose is quite resistant to enzymatic attack.

Sugar containing raw materials

Any sugar containing fruits, fruit juices or extracts, sugar-containing effluents from canneries, sugar beet, sugar cane, sugar cane molasses, cheese whey or sweet sorghum may be used as raw materials.

Starchy raw materials

Cereal grains (wheat, rice, corn etc.), root crops (cassava), tubers (potatoes) are gelatinized by heating and then hydrolyzed to fermentable sugars by enzymes.

Cellulosic raw materials

Saw-mill residues, paper-mill residues, newsprints, potato peelings, rice straw, corn stover, peanut shells, cocoa and coffee husk, tobacco stalks and wheat straw contain α-cellulose, hemicellulose and lignin. These materials when used are delignified, hydrolyzed and then enzymatically treated with cellulases to convert them into fermentable sugars.

Microorganisms

Most of the commercial production is carried out with *Saccharomyces cerevisiae.* For production of ethanol from pentose solutions from corn stover hydrolysis and from spent sulfite liquor of wood industry *Candida utilis* is used and glucose is fermented with immobilized cells of *S. cerevisiae*. Many strains of *S. cerevisiae* readily produce 10-13% ethanol by volume in batch fermentation of molasses solutions with initial fermentable sugars 20-25%. Higher concentration of ethanol can be achieved in Fed-batch culture. The fermentation of whey requires the use of dairy yeast Kluyveromyces fragilis and *K. lactis*.

Nitrogen sources

Assailable Nitrogen sources such as ammonia, ammonium salts, urea, or amino acids are added in addition to phosphorus in the form of ortho-phosphate. Minor quantities of potassium, magnesium, calcium and trace elements are also added. The addition of vitamins is rarely required but thiamine has been often seen to accelerate the rate of fermentation. *S. cerevisiae* requires the presence of biotin. The best source of all required nutrients including the trace elements and vitamins is yeast extract.

Fermentation

Biochemical basis of fermentation: Yeasts, under anaerobic conditions, metabolize glucose to ethanol primarily by way of the EMP pathway. The overall net reaction involves the production of 2 moles each of ethanol, carbon dioxide and ATP per mole of glucose fermented. Therefore on a weight basis, each gram of glucose can theoretically give rise to 0.51g of alcohol. The yield attained in practical fermentations however does not exceed 90-95% of theoretical. This is due to the requirement for some nutrient for to be utilized in the synthesis of new biomass and other cell maintenance related reactions. Side reactions also occur in the fermentation (usually glycerol and succinate), which may consume up to 4-5% of the total substrate.

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ ATP$$ (Gay Lusac's Equation)

Glucose Ethanol carbon dioxide Energy

Modes of fermentation

Batch fermentation: Most of the production of ethanol is carried out on the same lines as Established hundreds of years ago. These methods are based on the simple batch fermentation of substrates inoculated with culture microorganism at temperature 20-30 °C, initial pH adjusted to 4.5 and the time varies between 36-72 hours. Final ethanol concentration is usually 10-16%. Batch technology has been preferred because of its ease of operation, low requirement of sterilization, use of unskilled labor, low risk of financial loss and easy management of feedstocks. It has its disadvantages also such as low productivity due to long turnaround times, expenditure of cooling as fermentation by this mode raises the temperature of the fermentation broth and initial lag in growth.

Continuous fermentation: In continuous fermentation, fresh medium along with inoculum (yeast)is fed to the fermenter and an equal volume of the fermented liquor is withdrawn from the fermenter for the recovery of ethanol and yeast. Continuous ethanol production eliminates much of the unproductive down time associated with the batch fermentation process. The microorganism inoculated in the continuous mode should always be in its exponential growth phase because this increases the time dependent productivity of ethanol. The rate at which medium in the fermenter is added or withdrawn is usually expressed as 'Dilution rate'. It is denoted by a letter 'D'. The time for which the fermentation solution remains inside the fermentor for fermentation is referred as 'Residence Time'. The dilution rate is the ratio of withdrawn liquid to the volume of total liquid in the fermentor. Hence, a dilution rate D = 0.1 indicates that one-tenth of the fermentor liquid is withdrawn per hour (or the residence time in the fermentor is 10 hours). In continuous fermentation residence time and dilution rate should be so adjusted to get higher ethanol production.

Cell recycle: In Cell recycle, yeast cells are separated from the withdrawn fermented solution by means of centrifugation or by any comparable process or settling and returned to the fermentor. This is done to overcome low cell density limitations and to maintain high cell concentration in the fermentor.

Vacuum fermentation: This is a novel fermentation process in which alcohol formed by fermentation is continuously removed from the system so that it does not become toxic to the microorganism. This process was described by Cysewski and Wilke in 1977, 1978 and by Ramalingham and Finn in 1977. The former took the advantage of high volatility of alcohol and conducted the fermentation at

temperature conducive for the growth of yeast and sufficient vacuum to boil off the alcohol while latter used vacuum lesser than what former had used and temperature 30 °C.

Aeration

Aeration is done to keep the cells under suspension and to maintain viability in cells. Therefore sufficient amount of air should be sparged into the fermentor so that dissolved oxygen concentration (expressed as Oxygen Tension) may not exceed its toxic levels.

Distillation

Distillation process may be divided into two stages i.e. Distillation proper and Rectification. In distillation proper, volatile components of the fermentor are separated from the insoluble solids and ethanol is concentrated to a distillate containing 30-96% ethanol by weight. In rectification, ethanol is separated from other volatile components such as aldehydes (acetaldehyde) and fuel oils (amyl alcohol 60-80%, isobutyl alcohol 15-30%, and n-propyl alcohol 0-10%) about 1 litre of acetaldehyde and 5 litre of fuel oil is produced for every 1000 litre of ethanol produced. The amount of the volatile component depends upon the starting material used for fermentation and fermentation process itself.

Ethanol has a lower boiling point than water. Concentration of ethanol is therefore always more in the vapor phase of ethanol solution in equilibrium with the water. When distillation of the water and ethanol mixture is carried out, ethanol concentration of ethanol is always more in the distillate. Water and ethanol form an azeotrope. (Azetrope is a mixture of volatile substances that at a given concentration has identical liquid and vapor compositions). For ethanol and water this concentration is 96% by weight of ethanol and the boiling point of ethanol is 78.2°C. Therefore initially easy separation of ethanol from the broth becomes increasingly difficult as concentration 96% by weight of ethanol is reached because volatilities of both water and ethanol become identical. Boiling at this point gives a vapor of same composition as that of boiling liquid and no further concentration of ethanol can be achieved.

Anhydrous ethanol may be obtained from 96% ethanol solution by forming a ternary azeotrope with benzene. This ternary azeotrope contains 74% benzene, 18.5% ethanol and 7.5% water and its boiling point is 68oC. Since this azeotrope contains more water than the ethanol water azeotrope, it can be distilled overhead and leaves anhydrous ethanol behind. Such a process usually consists of two steps simple fractionation to produce near azeotrope mixture followed by ternary distillation with an added chemical agent yielding anhydrous ethanol. If the extraneous material is less volatile than the feed, it is called a solvent and the operation is called extractive distillation. When the extraneous material is more volatile than the feed it is withdrawn with the overhead product and the operation is called azeotrope distillation. In addition to these methods water may also be removed from ethanol water azeotrope by the use of adsorbents, such as cellulose, or other dry plant materials (cracked grains). Inorganic desiccants have also been suggested for this purpose. An endless loop of yarn fibers is also used to remove water from ethanol water azeotrope.

By-Products of ethanol fermentation

During the fermentation of ethanol many by-products are also formed which must be removed effectively by the distillation system. The by-products more volatile than the ethanol are mainly aldehydes with acetaldehyde the principal compound. Methanol in small amounts is also formed by the hydrolysis of pectins present in the fermentation broth. The less volatile compounds formed as a by-product are Fusel oils which are a mixture of isomers of amyl alcohol 60-80%, isobutyl alcohol 15-30% and n-propyl alcohol 0-10%. Separation of by-products: The aldehydes are removed relatively easily due to the low boiling point of acetaldehyde as a distillation head product; however the separation is not complete. The residual aldehyde content in alcohol is typically 5-100 mg/L. Fuel oil, owing to its complex composition and limited solubility in water is more difficult to separate. Dry fuel oils boil in the range from 128-137°C and normally, being even less volatile than water, they remain in the bottom product. In the presence of water however, the boiling point of the mixture falls below that of the water alone as a consequence of their immiscibility. Fuel oil, therefore rises from the bottom of an alcohol-purifying column and concentrate in the middle of the column, from they are removed. In a continuous process fuel oil is removed by bleeding from 6th to 15th plate of the rectification section and then fed to the fuel oil separator.

Then specification of the industrial alcohol depends upon the intended use. Premium grade alcohol has low water content, low odor ratings and high permanganate time test values (presence of aldehydes).

a. **Definitions**

A. Denatured Spirit- Alcohol that is denatured by adding 10% methanol is called denatured alcohol or denatured spirit.

B. Rectified Spirit- Alcohol from which fuel oil has been removed is called rectified spirit or rectified alcohol.

C. Anhydrous Alcohol- Alcohol that has very less water content is called anhydrous alcohol.

D. Potable Alcohol- Alcohol that can be used for drinking purposes is called potable alcohol.

Different Types of Substrates for Industrial fermentations

Substrates are used as components of media suitable for the growth of microorganisms or the production of desirable microbial metabolites. Nutrient medium form the environment, in which microorganisms grow and from

which they draw substances necessary for the synthesis of cellular components and to produce energy that runs the biochemical machinery of the cell. Bio-chemically substrates may be classified as: 1) Simple-that contain the lowest amount of energy, e.g. water, carbon dioxide, oxygen, nitrogen and inorganic ions, 2) High-energy-contain highest amount of energy and 3) Intermediary substrates-that are formed during the transformation of simple substrates to the high-energy substrates. The function of the nutrient medium is to ensure the growth of the microorganism and has to contain individual substrates in a form best accessible to the microorganism, which usually means in solution. However, in some cases solid media are used and cells from gaseous phase may sometimes utilize some substrates. Besides major components medium also contains many other components necessary for microbial growth. Physico-chemical properties and medium composition adjustment help in the maintenance of the maximum rate and desirable direction of the microbial process.

Complex media are prepared from natural raw materials of animal or plant origin; the exact chemical composition of these media is usually not known and individual components can be determined only with difficulty. A complex medium is prepared by dissolving appropriate natural substances in water and is often further processed by hydrolysis, clarification complemented by additional nutrients, growth factors, and missing trace elements. Synthetic media are prepared by dissolving defined chemicals in distilled water to eliminate completely all minerals and trace elements usually present in un-distilled water. Complex media are most often used for technological purposes while synthetic media are employed only in special cases e.g. in the processing of substrates from the chemical industry or utilization of some waste materials. In laboratories usually synthetic media are used despite their numerous shortcomings because they are defined therefore it is possible to draw exact material and energy balances from changes in the levels of individual components. As a qualitative and quantitative combination of substrates, the medium must meet the microbiologist's demand. The consumption of substrates in the laboratory is very small and it is quite within the range of microbiologist's to use more expansive substrates that cannot be utilized on commercial scale due to certain reasons. According to state of the medium, media can be classified as: 1) Solid media. 2) Liquid media. 3) Gaseous media. The solid media are present in the solid state and they are used immediately on dissolving in distilled water. The liquid media are readily utilized as they contain more water. These can be converted onto solid ones or gels by the addition of certain thickening agents like agar-agar, gums, and gelatin etc. The gases also play an important role in the growth and multiplication of the organisms and they also have a profound effect on the production of metabolites. The main components of a medium are as follows: 1) Water. 2) Carbon source. 3) Nitrogen source. 4) Accessory substances. 5) Antifoam agents.

Water

Water is an indispensable component of a medium. It is much needed by microorganisms for fermentation process. It is also needed in other processes like isolation of the cells and products. In fermentation, we usually use tap water for the preparation of culture media, separation and washing of microorganisms. The quality of water used depends upon the process in which is used e.g. A water of low grade quality can be used for cooling purposes, whereas for the preparation of media and isolation of cells and products etc. a water of high quality i.e. Potable water or treated water is needed. The treated water can be produced by four ways: 1) By adjusting the chemical composition i.e. by removing salts or ions like iron, free chloride or chlorine, salts of carbonic acid and adjusting the hardness. 2) By removing the extraneous materials by sedimentation, filtration etc. 3) by removing microorganisms from the water i.e. by the processes like filtration, sterilization and disinfection. 4) By removing the colloids i.e. by the process of osmosis. The quality of water always fluctuates and can be controlled by various chemical and biological methods e.g. when levels of ammonia in water increases, the water is said to be polluted and has to be treated by various chemical methods or by growing certain organisms like ammonifying bacteria. Similarly when levels of salts of nitrous acid or nitric acid rise above the normal limits, growing denitrifying bacteria also controls them. When iron levels in water increases, it causes discoloration of microorganisms such as yeasts and inhibit the biosynthesis of various substances e.g. antibiotics. It may also produce corrosion like effect in water. The norms for high quality water are 20-100 germs per ml. The hardness of water is due to the presence of various salts of calcium and magnesium, which when rise above their normal limits, they lead to instability in product yields. In that case, water is deionized and appropriate portions of other salts are added. Such types of treatments are only possible in laboratory and at commercial scale; the cost of applicability becomes very high. The quality of ground water now days is further deteriorating due to the increases use of chemicals in agriculture and utilization of nuclear materials in the warfare. So the demand of water for the commercial purpose has further increases and further prospects of research in this regard are needed.

Carbon sources

They represent the main component of nutrient media quantitatively. They are utilized for the biosynthesis of cell materials and are also used as main energy source. Carbon is also used to build carbohydrates, lipids, fats and proteins etc. in the cell and it is derived from various organic compounds added in the medium. Some new types of substrates have also come into light such as synthetic alcohols, alkanes and many hydrocarbons. The nature of microbial process, product purity and metabolism of the inoculum microorganism may dictate the use of chemically defined carbon sources such as glucose, sucrose, lactose or starch as well as complex raw materials like molasses, sulfite liquors, wood hydrolysates, cellulose and raw materials of petrochemical origin.

Carbohydrates

They form a distinct group of organic compounds that are divided into three categories as monosaccharides, disaccharides and polysaccharides.

Monosaccharides

Glucose-In fermentation process, glucose is the most frequently used monosaccharide (Grapes contain 25-30% of glucose) but it has its own disadvantages like Catabolite repression and Pasteur effect. For industrial purposes glucose is obtained by the hydrolysis of starch both by chemical and enzymatic methods. Since it is rapidly assimilated therefore a continuous inflow of glucose is required in growing cultures for the biosynthesis of complex compounds. It is sterilized separately from the other components of the medium because at neutral pH and at high temperature it undergoes Maillard's reaction with amino acids to give dark brown colored solution. It is usually sterilized at pH 3.0 as a 30-50% solution (w/v); under these conditions the solution remains colorless. In microbial processes, the so-called "Hydrol", a waste product in the manufacture of pure glucose, can also be used as a substrate.

Disaccharides and oligosaccharides

1. Sucrose- It is obtained from sugar cane or sugar beet and is available in different levels of purity. It is much needed for the fermentation by fungi, yeast and in the fermentation of microorganisms those biosynthesize antibiotics, amino acids and organic acids.
2. Lactose- Lactose is present in the milk of mammals at the levels of 3-8% and is prepared by the evaporation of whey that remains after the processing of milk to butter. Only a few species of microorganisms can assimilate lactose; yeasts with a few exceptions do not assimilate lactose. *Penicillium chrysogenum* can assimilate lactose in low but in efficient levels. The main disadvantage of the lactose is its varying quality, which can be attributed to different milk processing operations.

Polysaccharides

These substances represent a suitable source for bacteria, yeast and fungi and are used in the biosynthesis of many products. They are macromolecular, forming colloidal solutions or exhibiting negligible solubility in water, for this reason they must often be converted into a soluble form before being used in nutrient media.

Starch: It is one of the most important polysaccharide both biologically and industrially. It is accumulated as a reserve substance in fruits, seeds, and bulbs of higher plants in the form of granule of different shapes and sizes. It is usually denoted according to its origin i.e. according to the plant from which it is manufactured i.e. Potato starch from potatoes, Maize starch from maize, Wheat starch from wheat, Rye starch from rye and Barley starch from barley. Starch granules are hygroscopic and do not changes at low temperatures while at high temperatures, they swell and change into starch gel. The gelation is usually performed at 100 °C in water. At 120-130 °C, the gel liquefies and may be easily degraded by enzymes. Starch is a mixture of two related polysaccharides, amylose (linearly arranged chain of α-glucosidically linked molecules of D-glucose with 1-4 bonds) and amylopectin (branched chain of D-glucose molecules bonded also -glucosidically by 1-4 and less often 1-6 bonds); one molecule contains more than one thousand glucose residue. Acid hydrolysis of amylose yields first maltose and then glucose. Enzymic degradation by amylases proceeds via amylodextrin and low molecular weight dextrins to maltose.

Cellulose: It is a structural polysaccharide, forms a substantial part of the plant cells and is found abundantly in nature. Isolation of pure cellulose is difficult because other natural substances like lignin, hemicellulose and waxes accompany it. For industrial purposes, cellulose is obtained from wood and straw of various plants. Recently an intensive research has been initiated concerning cellulose-degrading enzymes like cellulases. The cellulase is a multienzyme complex of three enzymes namely endoglucanase, exoglucanase and α-glucosidase that act synergistically to form glucose. A compound known as "Cadoxen" (25-30% ethylene diammine in water and 4.5-5.2% cadmium) has the capacity to disrupt the crystalline structure of cellulose, which renders it susceptible to rapid and total hydrolysis to form soluble products. Other agricultural wastes, which can be used as carbon sources are: Molasses from sugar industry, Barley water and its products from brewing industry, sulfite waste liquor from paper and pulp industry, acid-wood hydrolysates from wood and paper industry, vegetable oils and animal fat from oil industry and alcohol and hydrocarbons from petrochemical industry.

Molasses: It is a thick, syrup-like, viscous and dark colored liquid obtained as a byproduct in the production of raw and refined sugar from sugar cane or sugar beet (remains after the crystallization of main fraction). It is named after its source as Cane or Beet molasses. Cane molasses contains a high level of sugars

Barley and its products: Barley and its products such as malt, wort and malt extract. Malt is prepared by the germination of barley. During malting amylases and pectinases are only partially activated since the process takes place at low temperatures and in a medium containing only vegetable liquor. In the brewery malt is crushed, mixed with water and kept at a higher temperature; under these conditions the enzyme reactions proceed rapidly and completely as far as the degradation of malt starch is concerned. Wort is obtained from malt residue by filtration. It is used as a raw material for Beer brewing. After the addition of hops it is boiled and filtered. The resulting solution is hopped wort; after cooling it is inoculated by a culture of brewer's yeast and fermented. Malt extract is prepared from wort by filtration and evaporation; it is thick syrup containing 80% dry weight that may be vacuum dried to a powder.

Sulfite waste liquor-It is a waste product in the production of pulp from wood by the sulfite method. It is a brown-yellow liquid with a density of 5 to 90 B at 20°C. Its specific weight is 1.045 to 1.060. It contains 8 to 14% dry weight that consists of 18 to 20% minerals and 80 to 90% organic substances. The inorganic part is composed chiefly of sulfur dioxide, sulfite and calcium sulfate. A part of the calcium is bound to lignin as a calcium lignosulfate salt, which is water soluble in acidic media. Other inorganic compounds present in the liquor are salts of heavy metals such as copper, arsenic and lead. These heavy metals ions in their minute amount also affect the growth of microorganisms. The organic compounds found in the liquor i.e. lignin and carbohydrates, are released from wood during treatment with the sulfite cooking acid. A short cooking at low temperature yields solid pulp containing higher levels of hemicelluloses and lignin; therefore the resulting liquor contains lower amount of these substances. On the other hand long and intensive cooking at higher temperature produces soft pulp having very less lignin and hemicelluloses resulting in liquor of higher contents of these substances. The liquor further contains small amounts of volatile acids like acetic and formic acid. Further fermentation of sulfite liquor depends upon how much carbohydrates are released during cooking and pulping. The amount of carbohydrates varies according to the species of the pressed wood and technology of pulping. The composition differs in liquors from conifers and from deciduous trees. The total carbohydrates in the coniferous wood liquor usually include 30-40% mannose, 30-35% glucose, 10% galactose, 15% xylose, and 5-10% arabinose. In deciduous trees, the liquor contains 50% xylose, 15-20% arabinose, 10% methylpentoses, 10% mannose, and about 10% uronic acids. In addition they contain higher levels of furfural, which originates from pentoses during cooking at high temperature and pressure. The furfural affects negatively on the growth of microorganisms. The sulfite waste liquor cannot be used directly for fermentation in the form obtained from the cooker since 1) it contains a considerable amount of sulfur dioxide, which has to be removed. 2) Has fluctuating pH values, which can be adjusted with calcium carbonate or calcium hydroxide. 3) it may contain insignificant levels of assimilable nitrogen and phosphorus and these nutrients must therefore be added. Sulfite liquors are used especially for the production of ethanol and production of fodder yeasts.

Acid wood hydrolysates: The resulting pulp after the sulfite waste liquor has been removed still contains a considerable amount of carbohydrates other than cellulose. These are removed by low concentrations of acids, which hydrolyse hemicelluloses to soluble sugars; this process is called as prehydrolysis. Prehydrolysis removes only hemicelluloses and not cellulose in contrast to the complete hydrolysis that cleaves all the polysaccharides including cellulose. The prehydrolysis is carried out on deciduous wood, cereals, rape or rice straw, and reed grass. The straw prehydrolysates contain higher amount of pentoses and therefore are used for the production of fodder yeasts. Vegetable oils and Animal Fats-Majority of the vegetable oils are easily metabolized by microorganisms therefore they are used as a carbon sources in many microbial processes especially in the production of antibiotics. They are also often used as an antifoam agent. Fats on the other hand are solid at room temperature and their liquefaction requires high temperature. For this reason they are not so often used as substrates.

Alcohols-Synthetic alcohols, especially ethanol and methanol have importance as potential raw materials for microbial processes. Ethanol is available in sufficient quantities, has a reasonable price, standard quality and homogeneity. Its physico-chemical properties place it among surface-active substances; hence it increases the foaming of nutrient media. However, the foam is thin and facilitates a better distribution of air into liquid. Synthetic alcohol is easy to produce in pure form and is highly water-soluble. Its disadvantage is the presence microorganism inhibiting substances like crotonaldehyde. Methanol is also water-soluble and has similar properties like that of ethanol. In comparison with ethanol it has a higher volatility and toxicity. Hydrocarbons-Microorganisms can utilize almost all hydrocarbons to larger or some smaller extent. Bacteria can assimilate all hydrocarbons while yeasts can assimilate only some of them. The best carbon sources among hydrocarbons are n-alkanes. Alkanes can be divided in to several groups according to the number of carbon atoms they contain. These are: C1- C9 n-alkanes, C10-C20 n-alkanes and C20 and higher n-alkanes. C1-C9 n-alkanes-Methane is the most widely used alkane in this group because it is readily available. It is assimilated by most of the bacteria but not readily by yeasts. An amount of 1 Kg of methane yields 0.8 kg of dry cell matter. Due to their yield and narrow range of possible sources the alkanes of this group are not suitable for microbial industry. C10-C20 n-alkanes -The alkanes of this group are readily assimilated by almost all microorganisms including bacteria, yeasts, actinomycetes and molds. The yield of cell mass per consumed alkane of this group is 90-100%. C20 and higher alkanes-Assimilation of this group of alkanes is slower because alkanes of this group do not exist in liquid state at normal cultural temperatures.

Nitrogen sources

Substances containing nitrogen in various forms may serve as sources of nitrogen for microbial growth. The range of nitrogen assimilation is much wider than the carbon assimilation. Many microorganisms, especially lower fungi, utilize nitrogen from inorganic nitrogen sources like inorganic ammonium salts. Ammonium sulphate, the cheapest of these compounds is often used in combination with ammonium hydroxide and gaseous ammonia for continuous regulation of pH. Another very good source of nitrogen is urea; since it decomposes at high temperature is should be sterilized by filtration. Quality wise nitrogen

sources cannot match with the pure laboratory chemicals because of the economic reasons. Although many microorganisms assimilate inorganic nitrogen, they often grow faster in the presence of organic nitrogen sources. At present many suitable nitrogen sources of plant and animal origin are available as waste products.

Inorganic and synthetic organic nitrogen sources

Ammonium sulphate is readily soluble in water; the purest form used in microbial industry is technical ammonium sulphate that contains a minimum of 20.7% nitrogen. The so-called coke and gas sulphates are considerably contaminated with tar substances and are used only exceptionally as substrates. Diammonium hydrogen phosphate, which is used especially in yeast production, is a source of both nitrogen and phosphorus. Ammonia is used either in the form of 25% aqueous solution or in gaseous form. Urea is used only exceptionally in microbial industry i.e. in the production of fine and high-grade products, due to its relatively high price.

Natural sources of nitrogen

Corn Steep Liquor-It is obtained as byproduct in the production of maize starch. It contains biologically active substances that differ qualitatively and quantitatively according to the quality of the corn used and its technological processing. These substances are released into the corn steep liquor during corn steeping and some microbial processes, especially lactic acid fermentation participating in the release, occur during steeping process. Moyer and Coghil first used corn steep liquor in the Penicillin fermentation in 1946 and that enhanced the yield of antibiotic many folds. Corn steep liquor contains nitrogen substances, lactic acid, reducing substances and ashes with many trace elements. An important criterion of the quality of corn steep liquor is the ratio of amino nitrogen to total nitrogen, which should be about 0.4 i.e. approximately 2% of amino nitrogen dry matter. This ratio indicates the intensity of lactic fermentation during steeping. Another criterion is the content of amino acids, which should be higher than 20%.

Potato liquor and fermented extracts from bran and oil seeds-Sometimes liquor from potato starch industry and fermented extracts from bran and oil seeds are used as substrates in microbial industry. Potato liquor is obtained as follows: The liquid formed by pressing potato pulp in starch production is subjected to sulphuriation and lactic acid fermentation by Lactobacillus delbrueckii and carefully dried. Though it contains many reducing substances yet it lacks amino acids like arginine, glutamic acid, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, proline, threonine and valine. The fermented extract of bran and oil seeds is prepared as follows: Wheat bran and ground tobacco seeds are mixed with water and a small amount of lactic acid and superphosphate is added. After six days of lactic acid fermentation at 50oC the liquid is filtered and evaporated in vacuum. Yeast extract-It is available either dry or in the form of paste. The extract is prepared by autolysis or plasmolysis of Baker's or Brewer's yeast. To remove bitter hop substances, Brewer's yeast is washed at pH 6.5 to 7.0 prior to treatment. Yeast cells must be kept in a viable state during washing and filtration in order to prevent losses of cell components. Baker's yeast does not need washings. The autolysis is performed in a large vessel under continuous stirring at a temperature above 75°C. The yeast is then transferred to a vessel containing sodium chloride solution, which induces cell plasmolysis. It is then filtered or centrifuged to separate cell walls and membranes. The liquid is rapidly concentrated at 37oC to prevent degradation of thermolabile substances and also to prevent contamination. Drying the liquid form in an atomizer or spontaneously in vacuum makes the powered form of the yeast extract. Yeast extract is a mixture of amino acids, peptides, water, soluble vitamins and carbohydrates. Soybean and Peanut flour-Soybean flour is an important source of nitrogen. Its most important constituents are proteins 44-47%, fat 5.5-6.0% and phosphorus 0.62-0.65%. It is obtained by the extraction of oil from soybeans. Peanut flour is also obtained after the extraction of oil from peanuts. It's important constituents are nitrogen, fat and phosphorus. The composition of proteins is variable and depends on the source of raw material. Both peanut and soybean flours are used as substrates in the biosynthesis of antibiotics. Peanut flour is suitable for the biosynthesis of Penicillin and Lysine while Soybean flour is suitable for the biosynthesis of Chlortetracycline. Peanut flour is not suitable for the biosynthesis of Tetracyclines.

Antifoam agents

Media when are rich in natural substances; foaming occurs in the microbial processes. This foam poses difficulties when it is uncontrolled. The outflow of a large amount of foam from the fermentor may reduce the volume of the culture and may lead to contamination. Various antifoam devices and certain natural or synthetic antifoam agents are employed to control foam in a fermentation system. The effect of these substances is based on their ability to reduce the surface tension of the liquids. The term 'Antifoam' is used for the substances that are added before the foaming actually occurs in fermentation while the term 'Defoam' applies to a substance added afterwards to knock down the foam. The natural antifoam agents used are soya, rape, coconut, sunflower and mustard oils. Antifoam agents of animal origin are tallow and deodorized fish fat. Mineral oils may also be used. Another group of substances used are alcohols. The most common compound of this group is Octadecanol either pure or in combination with lard oil or mineral oil. Specially designed antifoam agents are also available; among them are silicon oils. The silicon oils are usually used in water based emulsions containing 10% silicon oil. They are most effective in bacterial or yeast cultures but are less effective in cultures of filamentous microorganisms. Since the microorganism does not assimilate silicon oils, that is why silicon oils are very efficient

and a single dose at the start of the fermentation is usually sufficient to prevent the formation of the foam. A similar effect is exhibited by polyalcohol's with molecular weight of 2000 and alkylated glycols. Phosphorus and magnesium are important compounds of nutrient agar medium since they participate in energy transduction, especially in reactions mediated by ATP. Microorganisms need calcium, potassium, sulfur and sodium; therefore are added to the medium for microorganisms. Though trace elements such as iron, cobalt, copper and zinc are indispensable, they are usually present as impurities in other components and in water. Enrichment by these trace elements is necessary only when analysis of synthetic media or raw materials indicate their shortage. A major element in media is phosphorus, which can be added both in organic and inorganic form. The compound most frequently used in industrial microbiology is Diammonium hydrogen phosphate that also serves as nitrogen source. Superphosphate is used in the production yeast; it is a mixture of calcium hydrogen phosphate and calcium sulphate with variable content. Growth Factors and Vitamins Growth factors and vitamins are usually present in natural sources of carbon or nitrogen compounds. When these natural substrates are used as the only source of vitamins, it becomes sometimes necessary to add additional growth factors and vitamins in a pure form. Vitamins of the B-complex category are necessary as growth factor in the production of Baker's yeast. The production of amino acids by auxotrophic mutants requires the presence of certain vitamins; shortage or excess of these substances in the medium affects the yield. Production of certain secondary substances is greatly increased when precursors of these substances are added into the medium e.g. when phenylacetic acid or phenylacetamide is added into the medium; biosynthesis of Penicillin G increases but when phenoxyacetic acid is added into the medium; biosynthesis of Penicillin V is enhanced.

Yogurt

Yogurt is defined as the end product of a controlled fermentation of high solids whole milk with a symbiotic mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in the ratio of 1:1 for the manufacture of high-grade yogurt. Among the various cultured dairy products it is unique because it is the only product in which acetaldehyde in relatively high concentration is desired as an essential flavor component. Similar sour milk products are called as 'Matzoon' in Armenia, Matsoni in Georgia, Leben in Syria, Egypt and Algeria and Matsurad or Jodda in Sycilly.

Forms of yogurt

At present yogurt is sold and used in various forms in different parts of the world. These forms are as follows: 1) Plain Yogurt (Set type and Stirred type). 2) Flavored yogurt without fruits (lemon or Vanilla). 3) Yogurt with fruit puree (French style or Swiss style or Continental style -Whole or sliced fruits are mixed uniformly throughout the yogurt and Sundae style -Fruits may be filled in the bottom of the cup or on top of the yogurt to be mixed by the consumer). 4) Soft frozen yogurt (Flavored and Unflavored). 5) Hard frozen yogurt (Flavored and Unflavored). 5) Frozen yogurt sticks. 6) Fluid yogurt drinks. 7) Fruit topped with yogurt 8) Spray dried yogurt products for confectionary, bakery and soups.

Yogurt ingredients

a. Dairy ingredients: The dairy ingredients for the preparation of yogurt include whole, partially defatted milk, condensed skim milk, cream, and non-fat dry milk. All the dairy ingredients should be of very high bacteriological quality. Mastitis milk, rancid milk, partially fermented milk; milk containing antibiotics and sanitizing chemical residues cannot be used for yogurt production. Since yogurt is a manufactured product, it may have variations from the quality standards established by the marketing considerations. Therefore it becomes necessary to standardize the milk used in the yogurt production. The milk fat levels in yogurt range from 1.0 -3.25%. Yogurt can be classified on the basis of fat present as: 1) Yogurt -contains minimum of 3.25% fat, 2) Low-fat yogurt -containing not less than 0.5% and not more than 2.0% milk fat, 3) Non-fat yogurt -contains less than 0.5% milk fat. In all the categories of yogurt, a minimum milk solid non-fat and minimum titrable acidity stipulated is 8.25% and 0.5% respectively. In order to standardize the milk solids non-fat, cream, partially skimmed milk, and skim milk alone or in combination, concentrated skim milk, non-fat dry milk, or other milk derived ingredients may be used. The milk-derived ingredients include casein, sodium and calcium caseinates, whey; whey protein concentrates alone or in combination.

Sweeteners

Sucrose is the major sweetener used in yogurt production. Sometimes corn sweeteners and honey may also be used. The level of sucrose in yogurt mix appears to affect the production of lactic acid and flavor in the yogurt. Sucrose concentrations above 4% inhibit the growth of *S. thermophilus*. Sucrose may be added in a dry, granulated, free-flowing, crystalline form or as a liquid of 67^0Brix. Commercial yogurt has an average of 4.06% lactose, 1.85% galactose, 0.05% glucose and pH of 4.15. In frozen yogurt 6% corn syrup solids are added. Non-nutritive sweeteners like calcium-saccharine, maltol, and sorbitol alone or in combination may be used for diabetics. Sometimes lactase is added to break the lactose of milk.

Stabilizers

They produce smoothness, body texture, gel structure, reduce wheying off or syneresis, and increase shelf life. Stabilizers function through their ability to form gel structure in water, thereby leaving less free water for syneresis. In addition, some stabilizers perform their action by forming a complex with casein. A good stabilizer should not impart any flavor, should be effective at low pH values, and should be easily dispersed in the normal working temperatures in a dairy plant. The stabilizers generally used are gelatin,

vegetable gums like carboxy methylcellulose, locust bean, carob, guar and seaweed gums like alginates and carrageen an. Sometimes agar-agar or pectin is also used. Calcium chloride is added to control whey separation.

Fruit preparations for flavoring yogurt

Fruit preparations are present at the level of 15-20% in the final product. Generally fruits are added to meet the market demand. In every type of flavored yogurt, the composition of basic fruit preserve remains the same while its pouring styles are different. Fruit preserve consists of 55% sugar and a minimum of 45% fruit which is cooked until the final soluble solid content is 68% or higher (65% in the case of certain fruits). Frozen fruits and juices are the usual raw materials. Commercial pectin, 150grade, is normally utilized at a level of 0.5% in preserves and the pH is adjusted to 3.0-3.5 with a food grade acid, viz. citric acid during manufacturing of the preserve. Calcium chloride and certain food grade phosphates are also used in several fruit preparations. In Fruit on Bottom or Eastern Sundae style yogurt, 59ml fruit preserve on bottom is followed by 177ml of inoculated yogurt mix on the top. No flavor or sweetener is added. It is incubated till pH becomes 4.2 and then refrigerated. In western Sundae style yogurt, the top yogurt layer contains flavors and colors while in Swiss style yogurt, yogurt and mixed fruit layers are mixed together.

Flavors and colors

Only certified flavors and colors should be used for the preparation of yogurt. It should meet the following requirements. It should 1) exhibit true color and flavor of the fruit when blended with yogurt, 2) be easily dispersible in yogurt without causing texture defects, phase separation or syneresis.

Microorganisms

The culture is specified as a mixture of Lactobacillus bulgaricus and Streptococcus thermophilus in 1:1 ratio. The inoculum culture can be regenerated from a lyophilized culture tubes on media containing milk as a component.

Microbiology of yogurt

Streptococcus thermophilus is commonly referred as coccus and *Lactobacillus bulgaricus* as rod. Equal numbers of both of these bacteria are desirable for the production of tartness and green flavor of yogurt. The fermentation is carried out within a temperature range of 35 -45°C, optimum being 40°C. During fermentation, both rods and cocci work together to produce yogurt. Rod shaped Lactobacillus bulgaricus grows first slowly but their weak proteolytic activity liberates sufficient amount of the amino acids (glycine and histidine) and peptides to stimulate cocci. Streptococcus thermophilus now grows vigorously to produce moderate amounts of lactic acid, acetic acid, acetaldehyde, diacetyl and formic acid. When the pH of the yogurt mix (initial 6.3-6.5) drops below 5.5, the rapid growth of cocci is arrested and that of rods is favored. Oxidation-Reduction potential of the system is depressed. Depletion of oxygen and the availability of the formate ion stimulate the growth of rods. Until the pH value reaches 4.2 Streptococci grow very slowly. Below pH 4.2 Lactobacilli grow rapidly and dominate the fermentation. The major portion of the lactic acid and acetaldehyde necessary for the characteristic flavor of yogurt is contributed by the lactobacilli but the second part of the fermentation is greatly aided by the initial metabolic activity of the coccus component. Yogurt should be cooled at pH value of 4.2-4.3 to obtain a high-grade product.

Flavors of yogurt

Typical flavor of yogurt can only be detected in plain yogurt because it is a product of bacterial symbiosis. The production of flavor depends upon the proportion of rods and cocci and their combined metabolic activity. The distinctive flavor is contributed by lactic acid (odorless) and by acetic acid, which are volatile and have strong odors. Milk components and other ingredients in the mix also play a role in providing a background flavor. To obtain a good flavor one should keep in mind that 1) starter strain should be selected with a view to obtain a good symbiosis 2) proportion of rods to cocci should be equal 3) rapid cooling should be done at pH 4.2. If the pH value falls below 4.2, in large tanks that require a long time to cool down to 10oC then the desired cocci to rods ratio and flavor balance is lost. The product so obtained will be too sour and coarse.

Characteristics of a good yogurt

1) The body of yogurt should possess a relatively high viscosity and should be firm and cohesive enough to be removed and eaten with a spoon. 2) There should be very little wheying off during normal handling for mixing, cooling, pumping and packaging. 3) It should have a smooth, rich texture free from lumps, granules or graininess. 4) There should be no gas packets, fissures or gassy effervescence.

Factors affecting body characteristics of yogurt

The factors are: 1) Fat and SNF concentration in the mix. 2) Stabilizers used. 3) Control over weighing and blending of ingredients in the mix. 4) Heat treatment of the mix 5) Concentration of protein by new process such as ultrafiltration. 6) Concentration of calcium and magnesium ions. 7) Type of starter culture used 8) Incubation conditions used. 9) Initial pH value of the mix. 10) Handling during pre and post incubation operation. 11) Sucrose concentration in the mix.

a. Defects and their causes in yogurt

1) Graininess -If coagulum becomes exceedingly firm before stirring, the finished yogurt tends to grainy. In addition, the use of rennet to obtain a good body invariably leads to graininess. 2) Coarse Texture -Disturbance of the yogurt mix just before the gelling stage gives rise to coarse texture. 3) Granular feeling in Mouth -Inadequate mixing of the powdered products causes a granular feeling in the mouth. 4) Gas packets and Fissures -These are caused by

the trapped carbon dioxide or hydrogen gas produced by contaminant flora like E. coli, Bacillus spp. and yeasts. 5) Slimy feeling in Mouth -Slime producing strains when used in starter culture would result in a mouth feeling similar to the white of eggs. 6) Off Taste and Off Odor -These develop due to faulty fermentation.

Kefir

It is a historic and old product from Caucasus region of Russia. Kefir grains are used as starter culture that can be reused several times. The kefir grains are gelatinous white or cream-colored irregular granules from the size of wheat grain to walnut. These are made up of a polysaccharide called 'Kefiran', which is associated with denatured milk protein. These are insoluble in water and swell up when soaked in water to form a jelly like product. Bacteria and yeasts are present within the folds of the grains and there may be some symbiotic relationship between the grains and microorganisms. Various kinds of microorganisms are involved in the kefir fermentation these include: *Saccharomyces kefir, Torula kefir, Lactobacilli, Streptococci, and Leuconostoc spp. Coliforms, micrococci* and spore forming rods contaminate the fermentation.

Method of preparation

Fresh cow's milk is pasteurized at 85 °C for 30 minutes. It is inoculated with kefir grains taken from the previous batch after cooling to 25 °C. The incubation at 23 °C yields a soft curd, which after being agitated forms a beer like foam and fuzziness from which kefir grains come upward with the evolution of carbon dioxide. Kefir grains are separated and washed in cold water. They are stored in cold water at 4 °C or are dried in a warm oven. The kefir grains have alcoholic, yeasty sour with tangy effervescent flavor. Fresh kefir grains have activity up to 8-10 days while dried grains may remain active for 8-12 months. They contain lactic acid 0.8%, alcohol 1%, carbon dioxide and flavor compounds (acetaldehydes, diacetyl and acetone).

Acidophilus Milk or Reform Yogurt

It is a product formed by fermenting milk with an authentic culture of *Lactobacillus acidophilus*. The organism has its unique features that it can survive in the severe conditions of intestinal tract of human beings, animals and birds. Its ability to initiate growth in, or form colonies on media containing bile salts should be used as a distinguishing characteristic. The strains of man and animals have certain differences like DNA of human isolates generally had lower G+C ratio than that of pig and chicken biotypes. The acidophilus milk has its therapeutic value in controlling intestinal disorders but its mode of action has not yet been elucidated.

Method of preparation

The acidophilus milk is an extremely sour product. The finished product contains very little, if any metabolic byproduct other than lactic acid. It is prepared from partially skimmed milk, which is heated at 120°C for 15 minutes. The heating at such high temperature denatures the proteins of the milk that releases some peptides required for the growth of bacteria. It is then cooled to 37-38°C and inoculated with 5% milk starter culture. The incubation at 37-38°C for 18-24 hours yields Acidophilus milk, which is quickly cooled to 5-7°C and bottled when final acidity of the finished product reaches 1.0%.

Bulgaricus Butter Milk Or Bulgarian Milk

It is prepared from cow or mare's milk, which is fermented by a pure culture starter of Lactobacillus bulgaricus. This is particularly consumed in various parts of the world particularly Balkan countries. The starter produces the required acidity and flavor. It can also be prepared from cow or buffalo's skimmed milk.

Method of preparation

The milk is heated at 95°C for 30 minutes and cooled to 37-38°C. It is then inoculated with 2% milk starter culture prepared with Lactobacillus bulgaricus. The milk is incubated until the acidity reaches 1.4%. The product is cooled to 7°C. After incubation a curd like smooth mass is formed that is diluted and churned. The butter is removed. Buttermilk left has its acidity in terms of lactic acid (0.25%). The final product lacks aroma but has a pleasant flavor. It can be used as a substituent for milk but it has very less sugar content. It has diuretic effect when taken in large quantities **(Flow Chart 3)**.

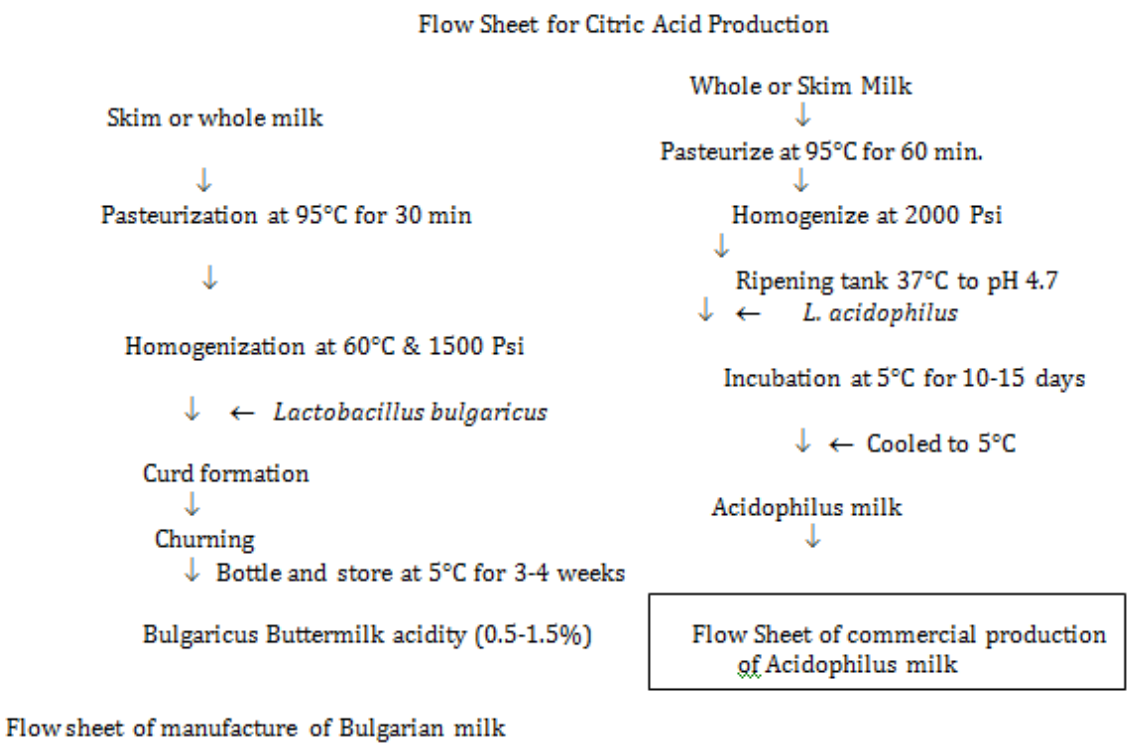
Role of heat treatment at high temperature in culture dairy products

Heat treatment at 85-95 °C for 30 minutes or equivalent is an important step in the manufacture of cultured dairy products. The heat treatment 1) produces a relatively sterile medium for the exclusive growth of starter culture. 2) Removes air from the medium to produce more conducive medium for microaerophilic lactic cultures to grow. 3) Effects thermal breakdown of milk constituents, especially proteins, releasing peptones, sulfhydryl groups which provide nutrition and anaerobic conditions for the starter. 4) denatures and coagulates milk albumins and globulins that enhance the viscosity and produce custard like consistency in the product.

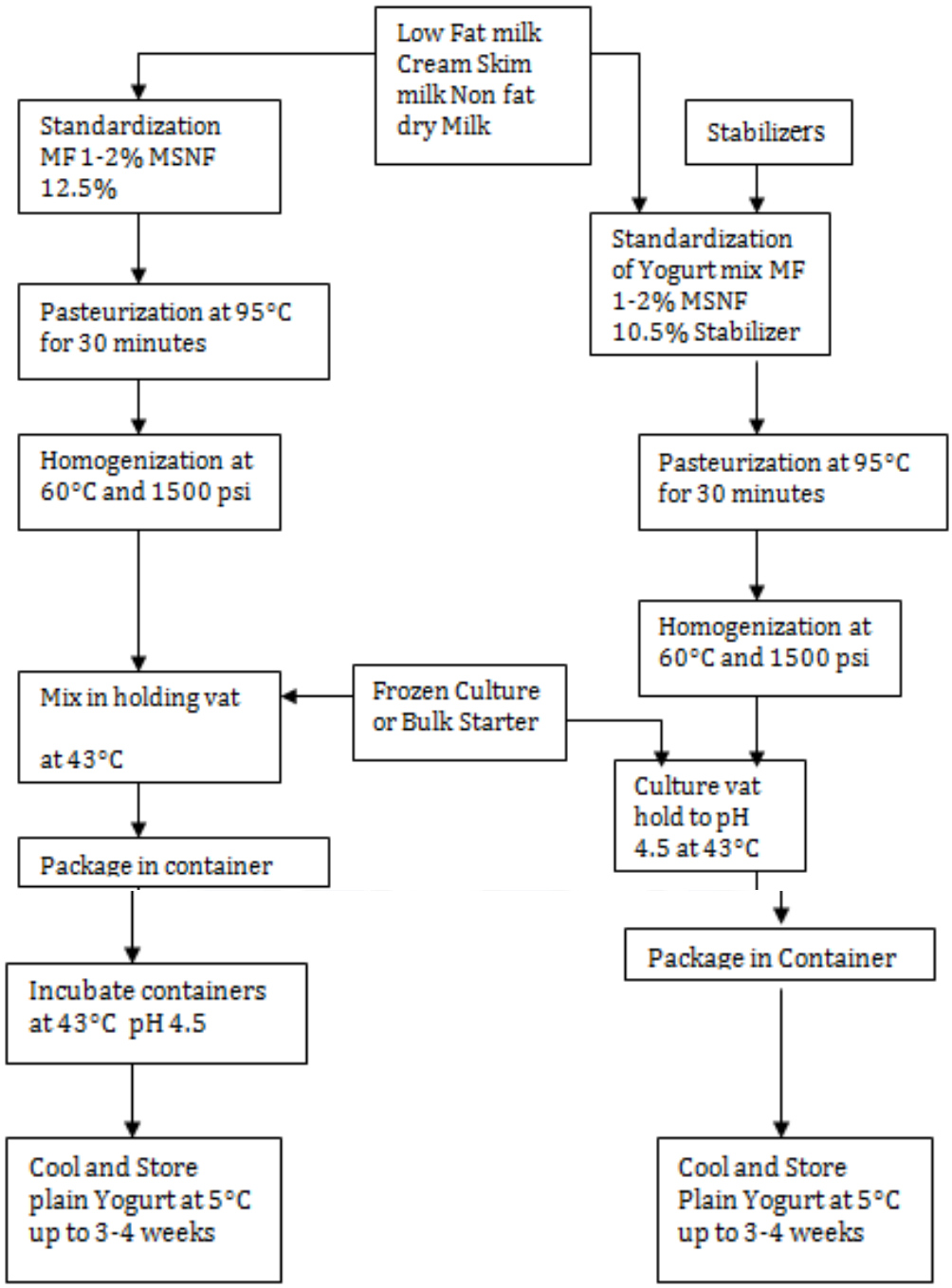
Flavor production in cultured dairy products

The characteristic flavor of cultured dairy products is produced by the activity of lactic culture by metabolic transformation of milk constituents. An important transformation of milk components into an essential flavor compound in cultured dairy products involves citrate metabolism. Milk contains an average of 0.2% citrate and it exhibits the greatest seasonal fluctuation (0.07-0.4%). Citrate is converted into 'Diacetyl' by flavor bacteria *Leuconostoc cremoris and Streptococcus lactis sub sp. Diacetylactis*. *Leuconostocs* ferment citrate only when there is sufficient acid development during the fermentation. Because leuconostocs do not produce much acid from the

lactose of the milk, therefore require an associative growth with lactic acid producing strain. *Streptococcus lactis sub sp. diacetylactis* on the other hand produces sufficient lactic acid, which required for the fermentation of citrate to produce diacetyls. The literature contains conflicting reports on the biosynthetic pathways involved for the production of diacetyls in aroma bacteria. Diacetyl synthesized by flavor bacteria in dairy products does not accumulate indefinitely. Once the concentration of diacetyl precursor, citrate falls below a critical value, the diketone is rapidly converted into a flavorless compound, Acetone. An enzyme called diacetyl reductase is widely distributed among flavor bacteria and other contaminating psychotropic bacteria commonly found in dairy environment, catalyzes conversion of diacetyl into acetone. Acetaldehyde is another important flavor compound but in cultured creamy butter, cultured buttermilk and cultured sour cream it is undesirable because it imparts a flavor defect referred to as green or yogurt flavor. It is a very important flavor component in yogurt and related products. It is primarily derived from lactose although other mechanisms for production of carbonyl residue are found among lactic acid bacteria. For example, metabolism of threonine and deoxyribonucleic acid results in acetaldehyde formation by bacteria. Many bacteria produce acetaldehyde; major ones are streptococcus lactis subsp. diacetylactis, Streptococcus thermophilus, Lactobacillus bulgaricus. Lactic Streptococci also produce a variety of carbonyl compounds that impart flavor important ones are: volatile fatty acids, formic acid, acetic acid and propionic acid. In koumiss and Kefir alcohol is an important component characteristic of the product. Torula kefir yeast and *Saccharomyces kefir* are responsible for the production of alcohol. One very important compound causing flavor problems in cultured milks is 3-methylbutanal. This imparts a malty flavor to cultured milks and ripened cream butter. Carbon dioxide plays an essential role in the flavor impact of cultured buttermilk, kefir and koumiss. The gas entrapped in the thickened milk provides lift, fizz, or effervescence to these cultured products. Carbon dioxide is derived from lactose by heterolactic bacteria. Fermentation of citrate by aroma bacteria also produces considerable amount of carbon dioxide. The minor metabolic products, although found in small or even trace amounts, may be important in maintaining a desirable flavor balance in cultured dairy products. Any shift in the flavor balance in cultured dairy products may result in the organoleptic perception of off-flavor. Rancid flavor present in milk is derived from straight chain free fatty acids up to 18-carbons by the action of lipase enzyme. Cream flavor is derived from isomeric 18-carbon unsaturated fatty acids by auto oxidation, 4-cis-Heptenol is produced. Butter flavor is due to β-lactones derived from hydroxy fatty acids by ring closure. Sharp sour flavor is produced by the fermentation of lactose to lactic acid by starter culture. Aldehydes, ketones, alcohols and esters combined to give milk a cowy flavor. Acetic acid contributes significant culture flavors. **(Flow chart 4, 5)**

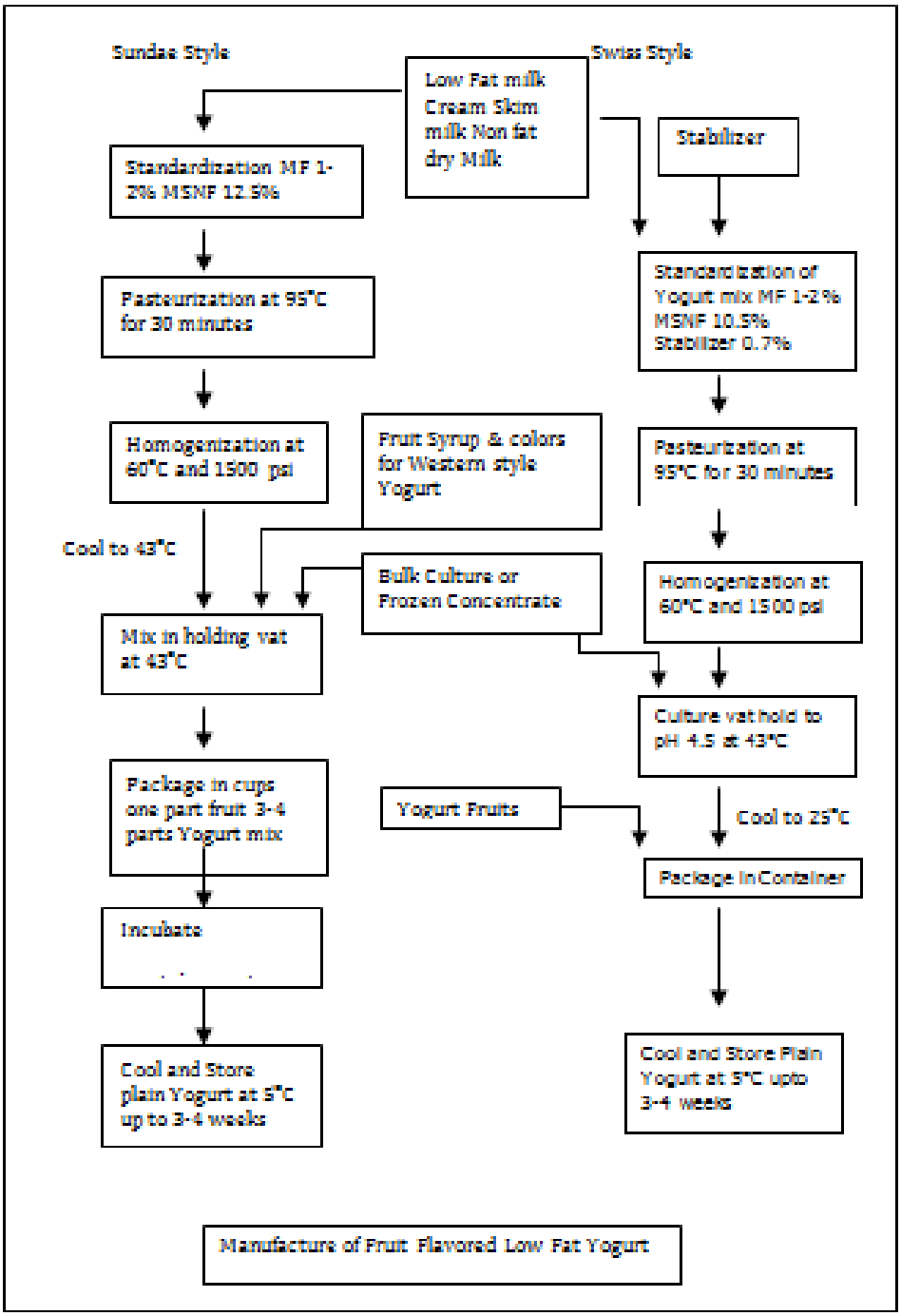


Flow Chart 3



Manufacture of SET and STIRRED TYPES of Plain Low Fat Yogurt

Flow Chart 4



Flow Chart 5

Starter cultures

Cultures used to start the fermentation in the preparation of butter, ripening of cream, souring of milk in cheese manufacture; preparation of various fermented milks and preparation of fermented cereal products are called “Starter Cultures”. They contain microorganisms that produce lactic acid from lactose, citric acid from various compounds or are involved in the fermentation of sugars to produce alcohols, diacetyls, or other flavor and aroma compounds. The microorganisms of the starters may grow in association or in succession to produce the desired product. Various purposes for which they may be used are:

Aroma production

Aroma producing microorganism is Streptococcus lactis that usually grows in fermented milk products. It produces lactic acid from lactose and also ferments citric acid to produce acetic acid, carbon dioxide, acetyl methyl carbinol, diacetyls and 2,3-butylene glycol.

Butter production

During butter ripening Streptococcus lactis produces lactic acid, volatile acids as acetic acid, propionic acid and carbon dioxide. Some neutral 4-carbon compounds like diacetyls, acetyl methyl carbinols and 2,3-butylene glycol add to the flavor and aroma.

Batter fermentation

In case of idli, dosa, jalebi, vada and bhatura, culture of the previous batch is added as starter culture that starts the fermentation. The starter causes leavening and acidification of the batter that produces a pleasant flavor in the batter.

Preparation of fermented milk products

In fermented milk products preparation, the flavor and body is so related with the starter that without good starter culture, the product is sure to become unsatisfactory.

Dahi or curd

Dahi is the most important sour milk product used throughout India. Dahi is the common term used in India for any kind of sour milk. But sour milk from different parts of India varies very much in quality, consistency and microflora. Dahi of Northern India is much thicker than in Southern India where dahi is generally used in diluted form. In some parts of India Cane sugar is added to milk to prepare sweet dahi. Sweet dahi is liked very much in Northern parts of India. Dahi sold in the market is prepared from fresh raw milk, but it is advisable to prepare dahi from boiled milk. Dahi prepared from fresh raw milk is fermented by natural flora i.e. no starter is added. When it is prepared from boiled milk, a little portion of dahi from the previous day is added and milk is allowed to curdle at room temperature. As dahi is prepared without special care regarding its mother starter, its quality varies from place to place and is never uniform.

Method of preparation of dahi: Dahi of good quality should be prepared in the following way: Good

quality whole milk or skim milk may be used for the purpose. Milk should be heated to 85 to 95 °C for half an hour or boiled for 10 minutes. It is then cooled to 45 °C. A small portion of the starter from good quality dahi is then inoculated into the milk. The amount of starter added will vary with the type of the starter, condition of the milk and incubation period. The milk is then incubated at temperature near about 40 ^{0}C. After 24 hours of incubation dahi of good quality may be obtained.

Microflora of dahi: Very little information is available regarding the microflora of this important

milk product. The samples of dahi from south India showed predominance of Lactobacilli. But samples of dahi from North India showed predominance of Streptococci. The species of lactic acid bacteria occurring most commonly in dahi include *L. bulgaricus, Streptococcus thermophilus*, *S. faecalis, S. lactis, L. casei and L. plantarum*, in order of their frequency.

Soy sauce: Soy sauce is a dark brown liquid with a salty taste and distinct aroma, which is made by

fermenting soybeans, wheat, and salt with a mixture of mold, yeast and bacteria. It is a seasoning agent used as a substitute for salt in the preparation of food as well as a table condiment. It enhances the flavor of the food and adds color into meats, sea foods, vegetables etc. The fermentation of soy sauce is essentially a process of enzymatic hydrolysis of proteins, carbohydrates and other constituents of soybeans and wheat to peptides, amino acids, sugars, alcohols acids and other low-molecular compounds by the enzymes of microorganisms. The brewing of soy sauce originated in China many centuries ago and later was introduced in Japan and other oriental countries. Soy sauce is consumed in almost every oriental country and has different names in different countries. It is known as Chiang-yu in China, Shoyu in Japan, Kecap in Indonesia, Kangjang in Korea, Toyo in Philippines and See-iew in Thailand. Japan leads the soy sauce industry in the world. It has not only the largest fermentation plants but also has the most advanced technology. Japanese shoyu is produced primarily in the areas near Tokyo and in Chiba prefecture. Japanese shoyu is mainly of three types: Koikuchi, Usukuchi and Tamari. Nearly 90% of the Japanese shoyu is of Koikuchi type, with dark reddish brown color and strong flavor and made from a nearly equal mixture of soybeans and wheat using Aspergillus oryzae hydrolytic enzymes followed by vigorous lactic acid and alcoholic yeast fermentations. It is pasteurized at a relatively high temperature. Ten percent of shoyu is Usukuchi type, light in color with maximum total nitrogen content 1.2%. Third type of shoyu is Tamari in which major portion is soybean and smaller portion is wheat. The highest quality shoyu is made by fermentation but second and third grades contain some portion of chemical hydrolysates.

Preparation of soy sauce: Soy sauce is prepared by fermentation of a mixture of soybeans and

Cereal usually wheat and salt. However, in recent years, defatted soybean meals and flakes have taken place. Today, more than 90% of soy sauce production in Japan is from defatted soybean products. In addition to the fermentation process, a chemical process in which acid hydrolyzes the proteins and carbohydrates is also being used in some western countries. In this method acid hydrolysis usually results in a complete breakdown of the substrate than enzyme hydrolysis. However, acid hydrolysis cannot perform many other specific reactions or interactions of hydrolyzed products as carried out by the multiple enzyme system produced by molds, yeasts and bacteria. That is

why chemically hydrolyzed product does not possess the flavor and odor of the soy sauce prepared by fermentation process.

Treatments of the raw materials

Soaking soybeans: In preparation for fermentation, selected soybeans are cleaned by ashing and soaked for 10-15 hours at room temperature. The water is changed every few hours to prevent acidification by bacteria. Weight of beans should increase 2.1-2.5 times during soaking.

Cooking soybeans: Hydrated soybeans are cooked for 1 hour in steam at 10-14 lb/sq in. ina rotary cooker (capacity 1 ton). They are then cooled rapidly. When defatted soybean meals or flakes are used, they are first moistened by spraying with water amounting to about 130% of soybean weight and then are steamed at 13lb/sq in. for 45 minutes.

Roasting wheat: Whole wheat or wheat flour is essential for production of typical Japanese

shoyu flavor. Usually low protein flour is used. Wheat is roasted in sand for several minutes at 170-180 °C and then the grains are crushed into 4-5 pieces in a roller mill. The roasting process adds flavor and color to the resulting soy sauce and in addition destroys surface microorganisms and facilitates enzymatic hydrolysis.

Effect of addition of wheat to the fermentation mixture: According to Yokotsuka (1964),

the addition of wheat to the fermentation mixture serves several functions. Firstly, the mold grows better and produces more enzymes on a mixture of wheat and soybeans than on wheat or soybeans alone. Secondly, the addition of roasted, crushed wheat to the cooked soybeans would minimize the growth of undesirable bacteria i.e. moisture of cooked soybeans is 60%, which is ideal for bacterial growth whereas moisture of soybeans and wheat mixture (1:1) is about 45%, which is adequate for mold growth but not for bacteria. Thirdly, wheat serves as a precursor of sugars, alcohols, organic acids, and flavor compounds. Lastly, wheat is rich in glutamic acid.

Addition of salt: Commercial salt is generally preferred for making soy sauce because it may carry an inoculum of halophilic and halotolerant bacteria and yeasts. Salt is added in such quantities that it prevents spoilage and/or food poisoning bacteria and permits the development of flavor and aroma forming bacteria and yeasts.

Role of salt: In addition to giving a salty taste, sodium chloride acts as a preservative and also has a selective action on microorganisms that grow in the fermentation substrate.

Koji and tane koji

Koji is a Japanese name given to a preparation consisting of mold growth on cooked cereals and/or soybeans. Koji serves as an enzyme source for converting complex plant constituents to simpler compounds. Koji is prepared by adding a koji starter culture called “Tane Koji” in Japan in cooked cereals and /or soybean substrate. Different types of tane koji (soy sauce koji, miso koji) are available for commercial use in making soy sauce, miso and others.

Preparation of tane koji: Tane koji is prepared by using naturally selected or mutant strains of *Aspergillus oryzae or A. soyae* to give desirable starter for a particular fermentation. Although strains used for preparation of koji starter are different, the method for preparation is similar. Polished rice is soaked in water overnight, drained, steamed for 1 hour and mixed thoroughly with 2% wood ash as a source of trace elements. The mixture is then inoculated with spores of selected strains of A. oryzae and spread out on trays in layers approximately 1.5 cm deep. And covered with a moistened cloth to favor the growth of mold mycelium. After incubation at 30 °C for 5 days, the rice is well covered with mycelium and with green to yellowish green spores of A. oryzae. The spores are harvested, dried at 50 °C and stored at 15 °C. A koji starter is usually composed of a blend of spores of different strains in a definite proportion, so that various enzymes are produced in proper amounts during the preparation of koji.

Preparation of soy sauce koji: Soy sauce koji is prepared from a mixture of roasted wheat and steamed soybeans inoculated with a koji starter (soy sauce Tane Koji) consisting of selected strains of Aspergillus oryzae grown on polished rice. Inoculated soybean and wheat mixture is placed in wooden or stainless steel porous trays of depth 30-40 cm and several meters in length and width. The trays are then incubated at 25-35 °C. Aeration and moisture is carefully controlled. This step is completed in 45 hours because this prevents the development of Mucor spp. and bacteria and enhances the development of proteolytic enzymes. The end product is called Soy sauce koji, which is a mixture of fungal hydrolytic enzymes on soybean wheat mixture substrate. Soy sauce koji of superior quality has a dark green color, pleasant aroma, sweet but bitter taste and high protease and amylase activity.

Fermentation

The soy sauce koji is mixed with 1.2-1.5 volumes of salt brine (23% w/v salt) to make mash called “Moromi” in Japan. Moromi is fermented in large concrete tanks or wooden vats for 8-12 months. Since koji is not prepared under aseptic conditions, one would expect the presence of yeasts and bacteria in moromi. However pure cultures of Pediococcus soyae, Saccharomyces rouxii and Torulopsis spp. are added to the mash at the start of fermentation and at one month after the start of fermentation to accelerate the fermentation and to improve the flavor of the final product. High salt content ensures the development of flavor enhancing yeasts while lower brine to koji ration result in decreased utilization of total nitrogen. Traditional fermentation starts in April and takes a year to complete. In general low temperature fermentation gives better results, because the rate of enzyme inactivation is slow and enzymes remain active for a longer time.

Pressing: The matured moromi is pressed in a hydraulic press at 100kg/sq cm. (1379lb) for 2-3days into a liquid part, known as raw soy sauce, and a solid cake. When whole soybeans are used as raw materials, soy sauce oil, consisting chiefly of ethyl esters of higher fatty acids, is produced during fermentation and appears at the upper layer of raw soy sauce. This oily layer must be removed and has no potential use. This is one of the reasons that defatted soybean meal is used instead of whole soybeans as raw materials in soy sauce fermentation.

Pasteurization and bottling: The raw soy sauce liquid is pasteurized at 70-80 °C in a kettle or heat exchanger, cooled, filtered to remove precipitates and stored. The final product is bottled for market. The bottles are usually made of plastic or glass and sometimes benzoic acid or propyl or butyl ester of p-hydroxy benzoic acid is added as preservative.

Benefits of pasteurization

1) Flavor, color and clarity is produced by the removal of oil particles mixed with heat coaguable substances 2) Inactivation of enzymes occur, which gives a stable product 3) Concentration of compounds like aldehydes, acetals, phenolic compounds, mercaptans, organic acids, pyrazines, furfurals, and β-diketones is increased 4) Because of the increase in phenolic compounds and organic acids resistance to spoilage by film forming yeasts occur.

Role of Mold in foods fermented by molds

Synthesis of enzymes: Molds, in these food fermentations synthesize enzymes that decompose complex compounds, including proteins, carbohydrates and fats into smaller molecules. At the same time, other compounds may be synthesized from the food substrates. These complex changes are accompanied by changes in the original properties of the raw materials. Taste, flavor, texture, color, palatability and other properties of the raw materials are usually modified in such a way that product becomes more attractive to the consumer. In addition to this general function of producing enzyme, in certain products the mold has a special role to play.

Mold growth: Mold growth on certain products contributes to the appearance of the food, which is desired by the consumer. *Neurospora spp*. provides oncom (fermented food) cake with a coating of its pink orange colored and powdery conidia. Rhizopus oligosporus covers tempeh cake with a clean white mycelium surface layer and additionally has the function of binding together the soybean into a solid, compact cake.

Synthesis of coloring compounds: The function of *Monascus purpureus* during fermentation of Angkak (fermented food) is the production of red colored compound monascorubin and a yellow pigment monascoflavin in soaked rice.

Protection of the product: Molds, which are traditionally used for the fermentation of oriental foods, have shown that they do not produce Toxins. But, on the other hand they resist accumulation of certain toxins which otherwise will be produced by other microorganisms in the food. This could be considered as a protection of the product against other harmful microorganisms. A good example of such a protective role is demonstrated by Rhizopus oligosporus, the mold species used for the production of Tempeh. This mold species does not produce aflatoxins. On the contrary, if aflatoxins are already present in the growth substrate, *R.oligosporus* could lower its contents to about 40% of its original content. In addition it was found that R. oligosporus inhibits growth, sporulation, and aflatoxin production by Aspergillus flavus.

Mold starters

The mold species that are traditionally used for fermentation of foods in different parts of the world belong to different genera. Species *of Rhizopus, Mucor, and Aspergillus* are used for the fermentation of foods throughout the orient, with the exception of Japan. In Japan, it is restricted to species of Aspergillus including A. oryzae and A. soyae. These species are used in Tane koji, which is used as starter culture for the preparation of soy sauce, miso etc. Tane koji is prepared by growing the mold on steamed rice. In other Asian countries, Ragi type starter cultures are in common use. Cultivating molds on cakes made of rice or wheat flour, which has not been steamed or cooked, makes these starters. The difference in preparing growth substrates for manufacture of two types of inocula is thought to be the cause of a natural selection of mold species which are developing in each of the type starters over many centuries, when they were produced with non-aseptic, traditional methods.

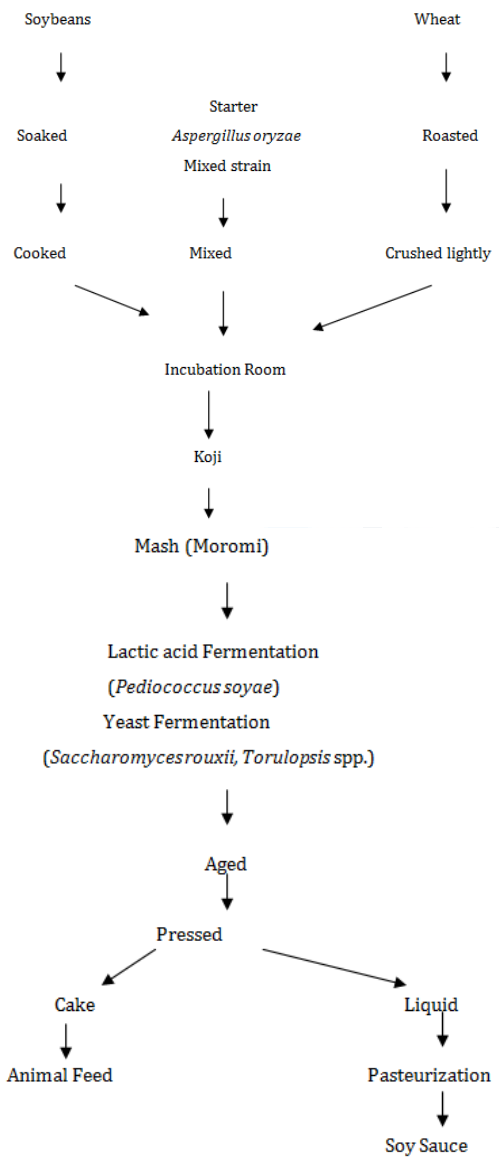
Yeast fermentations

When yeasts are involved in the fermentation process, production of alcohols improves the aroma of the product. In addition, alcohol at a certain concentration makes the substrate unsuitable for microorganisms, which may create undesirable properties in the product. Combined with the organic acids that are produced by lactic acid bacteria, the inhibitory effect of alcohol on undesirable microorganisms is increased. **(Flow Chart 6)**

Miso

Miso is a paste like product made by fermenting cereals, soybeans and salt with molds, yeasts and bacteria in Japan. This product is generally known as Bean Paste. It has the consistency of peanut butter, some smooth and some chunky and its color varies from light yellow to reddish brown. It has distinctive pleasant aroma resembling that of soy sauce, and it is typically salty (degree of saltiness may vary) and may sometimes has sweet taste. It is like soy sauce is used as a flavoring agent in cooking as well as table condiment and can be used in place of soy sauce. The product is known as Chiang in China, Doenjang in Korea,

Tao-Chico in Thailand, and Tauco in Indonesia. All mean bean paste. In Japan, there are many types of miso and they can be prepared by varying the ratios of soybeans to cereals, salt content, length of fermentation and addition of other ingredients such as hot pepper, which is very popular in China and Korea.



Flow Chart 6

Japanese miso can be categorized into three major groups as follows:

1) Rice miso -it is prepared from rice, soybeans, and salt 2) Barley miso -it is prepared from barley, soybeans and salt 3) Soybean miso -it is prepared from soybeans and salt. Each group can further be subdivided into white, light yellow, red according to color, and sweet, medium salty, and salty according to taste. Similar classifications can also be made according to the length of fermentation and fermentation temperature. This can be understood from the following table: (Table 1)

General method of preparation of Japanese rice miso

Cooking Soybeans: Whole soybeans are generally used for the preparation of miso, but sometimes dehulled soybeans or full fat soybean grits are also used for making white or yellow rice miso. Soybeans used should be of large size because the ratio of hull to cotyledons is lower in large sized beans. They should have high water absorbing capacity and when cooked under described conditions, the beans should be homogeneously soft with fine texture and bright color. To prepare the whole soybeans for fermentations, they are washed, soaked in water for about 20 hours at 16 °C and drained. The soaked beans are then cooked in water (white miso) or steamed at a temperature 11 °C for about 20 minutes in a closed cooker and slightly mashed **Table 2**.

Preparation of rice miso koji

For Rice miso koji preparation, polished rice is used. Since it is essential that the mold mycelium quickly penetrates the rice kernels, the rice is soaked in water (15°C) overnight or until moisture content is about 35%. Excess water is drained off, and the soaked rice is steamed at atmospheric pressure for 40 minutes. After cooling to 35°C, tane koji (koji starter culture), which is a blended mixture of several different strains of A. oryzae, prepared as described in the soy sauce preparation, is sprayed over the rice and mixed well. The inoculated rice is then incubated in a temperature and humidity controlled room. In about 40 -48 hours at 30 -35°C. The rice is completely covered with white mycelium of the inoculate culture. Harvesting is done while koji is white and before any sporulation has occurred. At this time, koji has a pleasant odor, lacks any musty or moldy odors, and is quite sweet in taste. The koji is removed from the fermentor and mixed well with salt to stop any further development of the mold.

Yeast and bacterial fermentation

Next fermentation is carried out under anaerobic conditions by yeast and bacteria. Cooked and slightly mashed soybeans are mixed with the salted koji and inoculated with a starter culture containing pure culture of Yeasts (Saccharomyces rouxii, Torulopsis spp.) and bacteria (Pediococcus halophilus, Sterptococcus faecalis). Miso from the previous batch can also be used as an inoculum of yeast and bacteria. Sufficient water is added to bring the moisture content equal to 48%. The mixture, now known as Green Miso, is thoroughly blended and tightly packed into a vat or tank for fermentation at 25 – 30 °C. During the fermentation period, the green miso is transferred from one vat to another at least twice to improve fermentation conditions. Fermentation time varies widely depending upon the type of miso. It is one week for white miso, 1 -3 months for salty miso and one year for soybean miso.

Table 1

Soybean : Rice : Salt	Type	Color	Taste	Fermentation Time	Fermentation Temperature
100 : 200 : 35	White miso	Bright light yellow	Sweet	2-4 days	50 °C
100 : 60 : 45	Salty miso	Light yellow	Salty	30 days	30 –35 °C
100 : 50 : 48	Red salty miso	Yellow red	Salty	60days	30-35 °C

Table 2

Protein	Isoelectric point	Protein	Isoelectric point
Egg albumin	~ 4.6	γ_1-Globulin	6.6
Haemoglobin	6.8	Lysozyme	11.0
Pepsin	~ 1.0	Myoglobin	7.0
Serum Albumin	4.9	Chymotrypsinogen	9.5
β- Lactoglobulin	5.2	--	--

Aging and packaging

At the end of fermentation, the fermented mass is kept at room temperature for about 2 weeks to ripen. The aged product is then blended mashed, pasteurized (60 – 70°C for 30 minutes), and packaged. Traditionally, miso is sold in wooden kegs of various sizes. Presently, it is sold in sealed polythene bags or tubes. For packaging into the plastic bags, miso must first be pasteurized at 60 –70°C for 30 minutes to prevent swelling. Sorbic acid or its potassium salt is also added at a level of less than 1 gm/kg of miso **(Flow Chart 7)**.

Dehydrated miso powder

This product has become increasingly popular. The dehydration is carried out by freeze-drying process. Dehydration process does not affect the flavor of the product. It has its potential use as an ingredient in the instant mix products**. (Flow chart 7)**

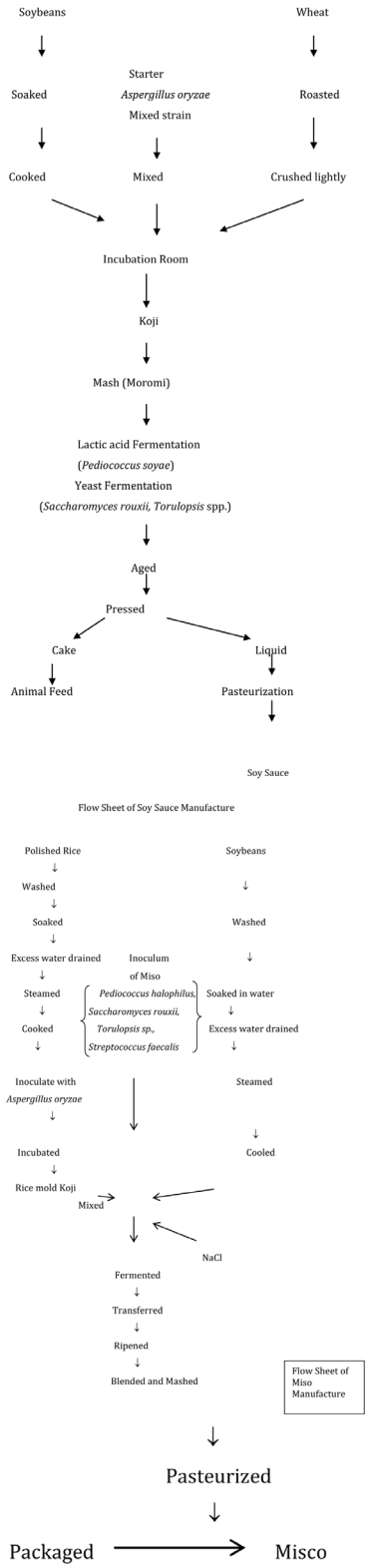
Tempeh

Tempeh or Tempe kedelee is a cake -like product that originated in Indonesia and is widely consumed in the regions of Malaysia and Indonesia. It is prepared by fermenting dehulled and briefly cooked soybeans with a mold, Rhizopus; the mycelia bind the soybean cotyledons together in a firm cake. The raw tempeh has a clean, fresh, and yeasty odor. When sliced and deep -fat fried, it has a nutty flavor, pleasant aroma, and texture that are familiar and highly acceptable to almost all people around the world. Unlike most of other fermented soybean products that are used as flavoring agents or relishes, tempeh is used as a main dish and meat substitute in Indonesia. It is easy to cook and does not possess the beany flavor of soybeans that most people find unpleasant. Because of its high protein content and universally acceptable taste and texture, it can be a potential source of low -cost proteins.

Preparation of tempeh

Microorganism: The mold used for tempeh fermentation was reported earlier to be Rhizopus oryzaebut in Indonesia, a strain identified as Rhizopus oligosporus saito NRRL 2710 is considered better producer of a good product. This strain is characterized by sporangiospores showing no striations and being very irregular in shape under any condition of growth. The sporangiospores are short unbranched and arise opposite to rhizoids that are much reduced in length and branching. It's all isolates also show Chlamydospores. Rhizopus oligosporus is highly proteolytic, which is important in tempeh fermentation because of the high protein content of the substrate. Two proteolytic enzyme systems were observed in the fungus; one has an optimum pH at 3.0 while the other at 5.5. Both the enzyme systems have maximum activities at 50 – 55 °C and are fairly stable at pH 3 -6. They rapidly denature at pH below 2 or above 7. In addition to high protease activity, the mold possesses strong lipase activity, low amylase and no detectable pectinase activity.

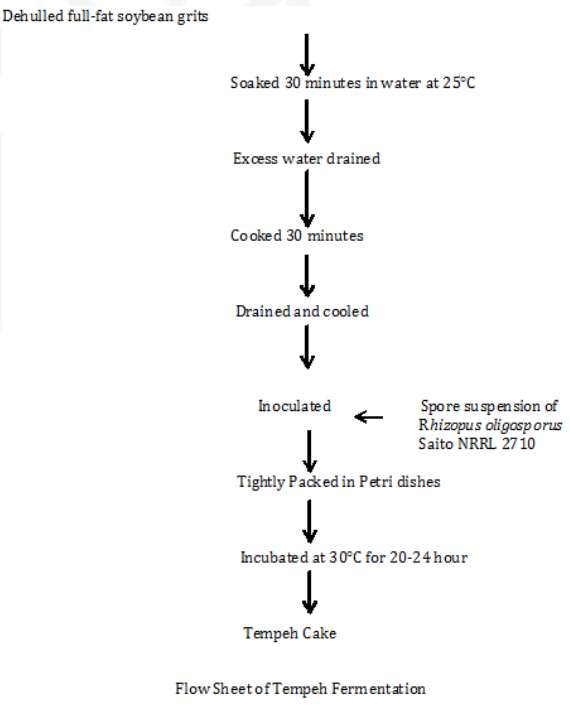
Preparation of soybeans for fermentation: The Full -fat soybeans are soaked in water overnight at room temperature. The soybean cotyledons can be mechanically cracked into 4-5 pieces, so that they absorb water easily and this also reduces the soaking time from 20 hours to 30 minutes. They are dehulled by hand or by a simple roller mill. The grits of beans are boiled in water for 30 minutes. They are then drained and spread to cool and surface drying.



Flow Chart 7

Fermentation

A pure culture of *R. oligosporus* spore suspension (prepared by adding a few milliliters of sterilized distilled water to the slant culture) already grown on potato dextrose agar and incubated at 28 °C for 5-7 days is mixed with the cooled and surface dried soybeans at the rate 106 spores/100gm of cooked soybeans. The inoculated soybeans are tightly packed into an appropriate container (petri dish) for incubation to obtain a final product in which a white mycelium developed abundantly but black spores are minimal. Rhizopus oligosporus like many molds require air to grow, but it does not require as much aeration as many other molds. In fact, too much aeration causes spore formation and may dry the beans resulting in poor growth. Therefore, it is important to properly pack the beans for fermentation. Many workers have successfully tried different types of packing materials. These are petri plates, aluminum foils or metal trays, with perforated or woven mesh bottoms and covers or perforated plastic film covers or perforated plastic bags and tubings. When the fermentation is complete, the beans are covered with and bound together by white mycelia. Thus raw tempeh looks like a firm white cake and has an attractive and slightly yeasty odor. Prolonged fermentation often causes the product to become obnoxious due to the enzymatic breakdown of proteins **(Flow Chart 8)**.



Flow Chart 8

Idli

Idli is a breakfast food in most parts of India especially popular in southern India. It is acidified and leavened through fermentation by hetero fermentative acid bacteria rather than by the activity of the yeast. It is closely related to sourdough bread of the western world, but it does not depend upon wheat or rye as a source of protein to retain the carbon dioxide gas during leavening. The importance of idli lies in 1) its high degree of acceptability as a food 2) its protection against food poisoning and transmission of pathogenic microorganisms, because of acidity and 3) the fact that idli fermentation can be used in many parts of the world using various combinations of cereal grains and legumes to produce acid, leavened bread, or pancake like products 4) No wheat or rye flour is needed. Idli is a small, white, acid leavened, and steamed cake made by the bacterial fermentation of a thick batter made from carefully washed rice (Oryza sativa) and dehulled black gram dhal *Phaseolus mungo*. Idli cakes are soft, moist and spongy and have a desirable sour flavor. It is served like a pancake with butter, honey, and jam or with other sauces. It can also be consumed directly "out-of-hand" following steaming or the cake may be deliciously flavored with fried mustard seeds and chopped coriander leaves. The unflavored cakes are eaten with chutney and /or samber, a thin-spiced soup of dhal and vegetables.

Details of manufacture of Idli

Ingredients: Idli preparation contains a number of different ingredients these are 1) Rice 2) Black gram dhal 3) Salt 4) water. Dehulled soybeans or Bengal gram can be used as a substitute for black gram dhal and a number of cereal grains can replace rice. However, there may be marked change in the texture and flavor when using substituted materials. It has been reported that rice variety and its physical characteristics are very important to produce a good quality idli. White Kar and IR20 varieties of rice have given much better performance in the production of idli, especially the White Kar variety because of its high amylose content, low amylopectin content better gelatinization, and better water uptake ability.

Proportion of cereal to legume

Ordinary idli consists of three parts rice and one part black gram dhal plus salt to taste. Kancheepuram idli is prepared from one part rice and one part black gram dhal plus cashew nuts, ghee, salt, pepper, ginger and cumin added to taste. Normally proportions of rice to black gram dhal varies from 4:1 to 1:4, the 2:1 being the best. It has been seen that when black gram dhal proportion is less than 25%, the steamed idli was hard and organoleptically unacceptable whereas when it is more than 50%, the product obtained is too sticky to be acceptable. Thus, not only can the ingredients be varied, but the proportions can also be varied within a wide range and still an acceptable product is obtained.

Soaking

Generally, the ingredients are soaked separately in water at room temperature for 5 to 10 hours before grinding to prepare the batter. Parboiled rice semolina can frequently be used while dry black gram dhal flour has been found unsuitable for the preparation of idli. If the idli batter is to be made without inoculation, it is essential that cereal and legume be soaked, ground with water and incubated at room temperature. Hot soak or hot grind will destroy the organisms essential for fermentation and, unless they are replaced by an inoculum, the fermentation will not proceed properly.

Proportion of water and salt to other ingredients

The amount of water added to the rice and dhal batter has varied from 1.5 to 2.2 times the dry weight of the ingredients. Batter should be rather thick for idli. Generally salt 0.8 to 1% is added to the batter as a seasoning before fermentation.

Fermentation time

Fermentation time varies from 14-24 hours, with overnight being the traditional time interval for the preparation of idli. The fermentation time must be sufficient to allow a definite leavening of the batter and allow for the development of pleasant acid flavor.

Inoculum

Ordinarily, the microorganisms developing during initial soak and then during the overnight fermentation are sufficient to leaven idli. The Central food technological Research Institute recommends adding one tablespoonful of buttermilk to each pound of its dry idli mix. The microorganisms that develop during overnight soaking are: *Leuconostoc mesenteroides, Streptococcus faecalis, Pediococcus cerevisiae, Lactobacillus fermentum, Torulopsis candida, Trichosporon pullutans, Torulopsis holmii* etc.

Incubation temperature

Ordinarily, the idli fermentation is carried out at room temperature. It generally means that temperature of 25 °C to 30 °C is probably an optimum temperature.

Steaming

The fermented batter is steamed as soon as the product has become leavened and acidified for 15-30 minutes at atmospheric pressure to get a soft, spongy and sour tasted and flavored idli. The fermented batter is poured into the cups of an idli steamer, which is placed in a covered pan and steamed until the starch is gelatinized and idli cakes are soft and spongy. They are generally consumed on the same day and there is no effort to preserve the product. The acid content of the product retards the growth of food poisoning and food spoilage causing microorganisms.

Microbiology of fermentation

In a study of sequence of microorganisms that developed during soaking of ingredients and subsequently during fermentation of the batter at 30 °C, it has been found that Leuconostoc mesenteroides and Streptococcus faecalis developed concomitantly and then continued to multiply following grinding. The number of these two species remained very high until 23 hours while in the later stages Pediococcus cerevisiae was also found. During this stage idli is being steamed and consumed. Thus, these two species are responsible for the acid production and leavening of the batter. The usual aerobic contaminants present on the ingredients are eliminated partly by careful washing and partly by acidic conditions produced by the fermentation. The fermenting microorganisms appear to be present on the ingredients and if the product has to be made daily, there might be some advantage in adding a bit of the freshly fermented batter to the newly ground batter. Yeasts like Torulopsis and Trichosporon can possibly leaven the batter if present in sufficient number, but it is highly unlikely that they produce the acid characteristics of idli.

Jalebi

To make jalebi, refined wheat flour (Maida), dahi and water are mixed into a thick batter and fermented for 14-16 hours. The fermented batter is deep fat fried in spiral shapes and immediately immersed in sugar syrup (600B to 750B) for a minute or two and eaten.

Microbiology of jalebi fermentation

Inoculum in jalebi ads from the natural microflora includes *Lactobacillus fermentum, Streptococcus lactis, Streptococcus faecalis, Lactobacillus buchneri and Saccharomyces spp*.

Changes during jalebi fermentation

During fermentation, pH decreases from 4.4 to 3.3, volume of the batter increases about 9%; both amino nitrogen and free sugar decrease.

Dosa

It is a thin crisp, fried, pancakes like staple food of southern India and is also gaining much popularity in other parts of the country. Like most of the fermented foods consumed in India and other Asiatic countries, dosa is prepared by natural fermentation. Dosa batter is very similar to idli batter, except that both the rice and black gram dhal are finely ground. The batter of dosa is thinner than the idli batter. Following fermentation, the dosa is quickly fried as a thin, fairly crisp pancake and eaten directly. The unflavored crisp pancake may be rolled onto a cooked mixed vegetable mixture and eaten with samber, which is a thin richly spiced soup of dhal and vegetables. It is an important source of protein and calories in the diet and nutrition of south Indians. Since is easily digested therefore it is often used as food for infants and invalids.

Method of dosa preparation

Ingredients: The ingredients used for dosa preparation are similar to as that of idli preparation i.e.rice, black gram dhal, salt and water. Rice may be substituted by wheat, bajra (*Pennisetum typhoideum*), maize, or kodri and black gram dhal may be substituted by sprouted peas, cowpeas (Vigna catjang), field beans (*Dolichos lablab*) or soybeans. Fresh groundnut oilcakes may also be substituted for black gram dhal.

Soaking and batter formation: Generally, equal quantities of rice and dehulled black gram dhal are soaked in water at room temperature separately for 5-10 hours. It is common practice that finely ground powders are used to prepare batter. The finely ground powders are mixed with water at temperatures ranging from 480 to 980 °C, best at 80 °C for the preparation of batter. Water is added in the range of 2.0 to 2.2 times the initial dry weight of ingredients to prepare a batter of viscosity desired for dosa. Salt is added from 0.8 to 1.0% as a seasoning before fermentation.

Fermentation Time: Traditionally, dosa batter is kept overnight for fermentation. The fermentation time should be sufficient to allow a definite leavening and acidification of the batter and to allow for the development of a pleasant acid flavor.

Inoculum: The natural microflora developed during the soaking operation and then at the overnight fermentation is sufficient to leave the dosa batter. However, fermented batter of the previous batch may also be used as a starter culture for fermentation. The microorganisms that develop during the overnight soaking are*: Leuconostoc mesenteroides, Streptococcus faecalis, lactobacillus fermentum*, *Pediococcus cerevisiae, Trichosporon pollutants, Trichosporon beigelii, and Candida kefyr* etc.

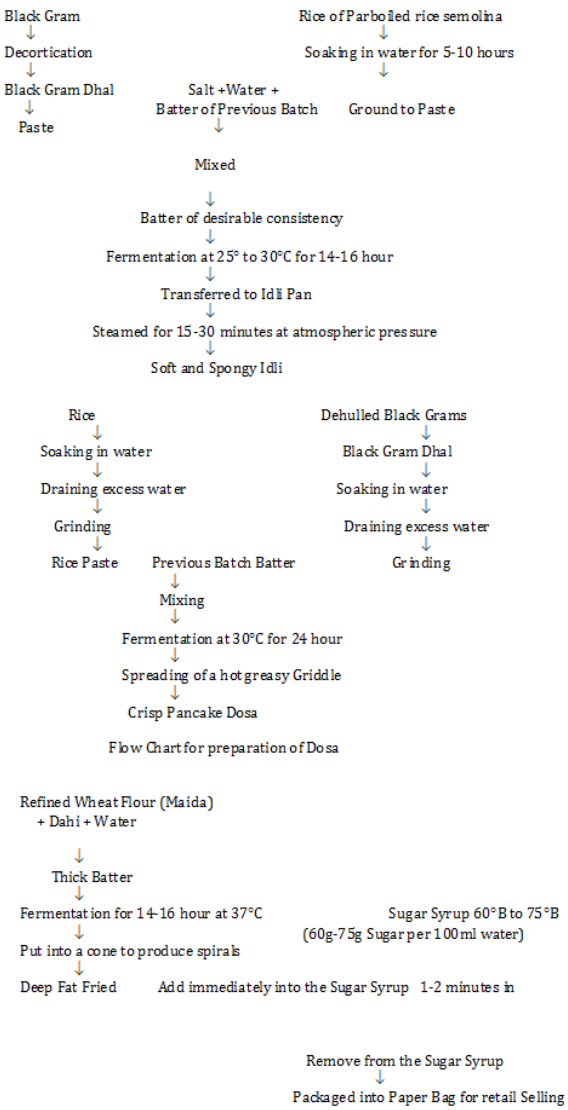
Incubation temperature: Ordinarily, the dosa fermentation is carried out at room temperature. In the tropics, this generally means a temperature of 25-30 °C.

Fermentation containers: In Indian homes, fermentation is customarily carried out in utensils that have sufficient capacity to hold the batter and clean to avoid excessive contamination. The containers are covered with a clean damp cloth to prevent the entry of insects.

Grinding of the Ingredients: Stone mortars are used for grinding the ingredients in Indian homes. These provide excellent control of the particle size in batter. The ingredients are very finely ground to a thin paste and then salt is added in it.

Harvesting and preservation: As soon as the batter becomes leavened and acidified, it is spread onto a hot and greasy griddle where it assumes the shape of a crisp pancake. The spreading of dosa on the griddle is an art that matures with practice. Sometimes a mixture of cooked different vegetable is poured onto the crisp dosa and the sides are rolled, which now becomes ready to be eaten with a spiced soup of dhal and vegetables popularly known as Samber.

Microbiology of dosa fermentation: Traditional dosa batter fermentation has revealed the Occurrence and role of several bacteria alone or in combination with yeasts in bringing about various biochemical changes but Leuconostoc mesenteroides appears in the fermentation early and brings about the leavening in batter. It is also along with Streptococcus faecalis involved in the acid production. These organisms appear to be present on the ingredients and develop during fermentation. Therefore it becomes an advantage that previous batch inoculum is used as a starter for fermentation. Yeasts, if are present in sufficient number can leave the batter but they have no role to play in acid production, which is characteristic of dosa batter fermentation. Pediococcus cerevisiae appear very late in the fermentation when the batter becomes ready to be fried on hot and greasy griddle. **(Flow Chart 9)**



Flow Chart 9

Fermented sausages

Sausages are cylindrically shaped, fermented, solidified, and heat stabilized emulsions formed by mixing together proteins, fats, water, salt and flavorings. Color, flavor and texture of sausages depend on the type of meat, which is mixed and comminuted together with ice, salt, spice flavorings, curing agents and selected meat trimmings, to form a sausage emulsion. The word “sausage” is derived from the Latin, salus; meaning salted or literally, preserved meat. The term “salus” undoubtedly was used by the Romans to denote meat preserved through the use of salt. Basically all sausages are comminuted meats. Products differ primarily because they are spiced in varied fashion and because of differences in methods of processing.

Classification of sausages

Sausages may be loosely classified into three general categories: 1) Fresh sausages. 2) Cooked or Smoked sausages and. 3) Dry Sausages. Some sausages, especially the dry and semi-dry types depend on bacterial fermentation of the production of their characteristic flavors, while in manufacture of some of the more common sausages such as frankfurters bacteria play no role. No distinct classification can be made based on spice formulae because basic spices are used in virtually all products.

Fresh sausages: Fresh sausages are always kept under refrigeration and are fried, boiled, or Cooke thoroughly before serving. They are prepared from selected cuts of fresh meats, principally pork, or beef that has not been previously cured. Examples of fresh sausages are fresh Pork sausages that are prepared selectively from pork meat and fresh sausages that may also contain a percentage of beef and other meats in addition to pork meat. Pork trimmings are put through a grinder, seasoning is added and mixed, and the product is stuffed into natural casings or sold in bulk.

Cooked or smoked sausages: These can be divided into two categories -1) smoked and un cooked and 2) smoked and cooked. Both these categories of sausages are prepared from cured meats and must be kept under refrigeration prior to preparation for serving. Smoked and uncooked sausages -include Smoked country-style Pork sausages that are subjected to mild curing and then placed in casings and smoked and cooked before serving. Other varieties like mettwurst, smoked country-style sausage and polish sausage, all contain beef and pork mixed in various proportions. Smoked and cooked sausages -these are prepared from both uncured and cured meats. They are processed by cooking and smoked after cooking. Examples of these are Frankfurters (60% beef and 40% pork), Bolonga (cured beef and pork), Berliner sausages (cured, coarsely ground pork and finely chopped beef), and German type Mortadella (Cubes of fat pork and pistachio nuts), Liver sausages (pork liver and gelatin), Blood sausages or Bluwurst diced, cooked fat pork plus finely ground cooked meat, blood and gelatin.

Dry sausages: Dry sausages are prepared by curing freshly comminuted meats added with Curing agents and spices for 2 to 3 days. They are then placed in casings and are processed by carefully controlled air-drying. When pork is used in such sausages, it is subjected to a light smoke in order to destroy live trichinae. The principal dry sausages are either salamis or cervelats. Salamis are generally more highly seasoned than cervelats.

Meat Ingredients for sausages: The meat ingredients used chiefly in preparing sausages are those parts of the animal that do not have a ready sale as such. These are classified according to their "water binding" properties i.e. their ability to retain moisture during thermal processing. Meats considered to have best binding properties are skeletal tissue from the beef animal, and include bull meat, shank meat, chucks and boneless cow meat. Medium binders are head meat, cheek meat, and lean pork trimmings. The meat with low binding properties usually contains large proportions of fat or are non-skeletal or smooth muscles. Examples are regular pork trimmings, beef brisklets, heart, giblets and tongue trimmings. Meat tissues classified as "Filler Meats" and considered to have little or no binding properties include ox lips and tripe, pork stomachs, skin, snout, lips, and particularly defatted pork tissue; their use in sausages must be severely limited if the quality of the product is to be maintained. The muscles of beef in sausages enhances flavor as well as contributes to color and texture. The beef muscles contain water-soluble nitrogenous extractives that act favorably on flavor. Fat in the sausages enhances flavor and changes the texture of the sausages. They become tender and juicy, but total fat in sausage must not exceed 50%.

Ice and salt: The addition of ice (moisture) assists in controlling the temperature of the emulsion while it is comminuted. Otherwise the chopping temperature will exceed 60oF, which will lead to instability of emulsion and promote bacterial growth. The moisture in the final cooked sausage should not exceed four times the meat proteins plus 10%. For fresh sausages that have not been heat-processed the limit is four times plus 3%. Water and salt make meat proteins soluble, which then stabilizes the fat globules in the sausage emulsion. Salt also adds to flavor.

Binders or fillers: Cereal flour, potato flour, soy flour, bread or cracker crumbs, milk powder, casein are some of the binders that are used both to hold together and to extend the meat ingredients in the sausages. The flours that are made from cereals as corn, durum wheat, and rye and from potato starch absorb water highly; however, they must not readily ferment when mixed with it. The soy flour with its low fat content enables making sausage flour high in protein content. Rice and cracker flours are also high in protein content. Binders or fillers improve color, provide better binding properties, improve slicing characteristics, change or improve flavor and reduce cost of the product. Their value depends on their ability to absorb moisture in the emulsion and retain it throughout heat processing. The proportion of fillers in sausages should not exceed 3.5%. When corn syrup and milk solids are used their proportion should not exceed 2%.

Curing agents: The common curing agents for sausage preparation are salt, sodium nitrite and/or nitrate and sugar. Three percent of salt on the basis of the meat ingredients is frequently used in sausages. Sodium nitrite and/or nitrate are usually added along with the salt to the beef portion of the sausage emulsion. The proportion of sodium nitrite may not exceed ¼ oz per 100 lb of meat; if sodium nitrate is used with nitrite, not more than 2 oz should be employed per 100 lb of meat. Sugar is added in many sausage products at a level of 0.5-1.0%. Other curing agents such as potassium nitrate (saltpeter), potassium nitrite, vinegar and flavorings are also added.

Seasonings: Variation in seasoning is one factor that is responsible for the large number of sausage varieties. These seasonings may be added as ground natural spices, extracted oils, and oleoresins, or a mixture of the two. Spices that are usually employed include allspice, black pepper, cardamom, cinnamon, coriander, garlic, mace, nutmeg, paprika and sage. Most seasonings for pork sausage contain 1.75-2.0 lb of salt, 6-8 oz dextrose, 4-5 oz pepper, 2-3 oz sage and 0.25 oz of ginger, Per 100 lb of meat.

Other additives: The other condiments used in meat products are pistachio nuts, mono sodium glutamate, ascorbates and isoascorbates. Pistachio nuts are used in headcheese, meat loaves and Braunschweiger. Monosodium glutamate is occasionally employed in pork sausage at a level of 0.1% to enhance flavor. Ascorbic acid or d-isoascorbic acid (erythrobic acid) is used at a level of ¾ oz or ⅞ oz of their sodium salts, in each 100 lb of sausage product. These are commonly used in smoked sausage to assure minimum color development and retention. Coloring matter and dyes, which are approved by the regulatory bodies may be mixed with the rendered fat, applied to animal and artificial casings, and applied to such casing enclosing products. The following coloring matter and dyes are acceptable 1) Natural coloring matters -alkanet, annatto, carotene, cochineal, green chlorophyll, saffron and turmeric 2) Coal tar dyes -all food certified dyes.

Sausage casings: Sausage casings may be natural casings prepared from some part of alimentary tract of cattle, sheep or hogs, or they may be cellulosic casings. These latter are frequently used on frankfurters and may be clear or colored.

a. Method of preparation of frankfurter sausage

Frankfurters are the most popular of all the sausage products. More frankfurters are consumed than any other type of smoked and cooked category of sausage; they represent 25% of all sausage sold. The meat formulation of ordinary frankfurters is 40-60% beef and 60-40% pork. Beef content includes Bull meat, boneless chuck, plates, hearts and trimmings while pork ingredients are filler meats such

as tongue, snout, lips and other byproducts in proportion not exceeding 20%; more will result unsatisfactory product. Fillers employed are dry skim milk, corn syrup solids and sometimes cereals. Total filler content should not exceed 3.5%. Meat used for preparation was pre-cured, but at the present time the curing agents are normally added at the time of chopping. Salt sugar and curing salts are added at the level of 3 lb of salt, ½ lb of dextrose, ¼ lb oz of sodium nitrite and 2 oz of sodium nitrate, for each 100 lb of meat. The more common spices and seasonings used are pepper, nutmeg, mace, cinnamon, mustard and garlic. After chopping and mixing, frankfurters are stuffed into casings and linked. They then held at refrigeration or ambient temperatures for varying periods of time prior to heat processing to permit completion of curing process. Ascorbic acid may be used to obviate the need for this holding period. The sausages are then heated and smoked. Immediately after smoking, they are cooked with a spray of hot water at a temperature of 77-82 °C. Simply raising the smoke house temperature finishes some frankfurters; such dry-processed products are usually given a brief hot water shower to plump the sausages and provide better peeling characteristics. The frankfurters are then cooled by cold water showering to an internal temperature slightly above ambient; the remaining heat is usually sufficient to dry the product prior to its placement in the holding cooler at 2-7 °C. The total processing time may be as short as 65 minutes (when the cure proceeds rapidly) or as long as 2.5 hours. Colored cellulosic casings may be used to add color, or dye may be mixed with the hot shower water, which must then be recirculated; occasionally frankfurters are colored by dipping. Certified coal tar colors can also be used. Frankfurters processed in cellulosic casings may have their casings peeled from them after processing to produce the product sold as skinless variety.

b. History of sausage

Sausage, one of the oldest forms of processed food, was developed some thousand years before the birth of Christ. It started by slow stages from simple process of salting and drying meats by the aborigines and was originated in part as a means of preserving meat that could not once be consumed. The American Indians combined chopped, dried meat with dried berries and pressed these ingredients into a cake for use when food was scarce. Similar drying of meat was a common place along the shores of the Mediterranean centuries before the rise of Roman Empire. The ancient Romans were extremely fond of a sausage made of fresh pork and white pine nuts, chopped fine and seasoned with cumin seed, bay leaves and black pepper. Salami is mentioned in Grecian literature of about 5th century B.C. Sausage was made and eaten by Babylonians (1500 B.C.). The fact that a city of Salamis existed on the east coast of Cyprus in Aegean Sea about 449 B.C. provides foundation for a supposition that salami sausage may have originated in this ancient Grecian city. The various types of sausages as we know today were developed in certain European localities because of local climate conditions. European sausage makers developed products that would keep under the climatic conditions of their particular area. Since artificial refrigeration and canning processes were unknown therefore sausage makers of Italy and southern France, developed dry sausage products. German and Hungarians produced definite types of dry sausages.

c. Action of microorganisms in sausages

Paulo and Smith (1977) have shown that Micrococci dominate the surface of stuffed sausage during the early stages of fermentation. These are killed at pH 5.5 and are not found in the sausage after heat treatment of drying. The results corroborated the research carried out by Sison (1967) in Philippines on the native chorizo-type sausages. The major functions of micrococci during the fermentation are the reduction of nitrate to nitrite and the production of catalase. Lactic acid bacteria rarely reduce the nitrates to nitrites. Lactic acid bacteria were also found in the fermentation. The activity of the lactic acid bacteria is the conversion of sugars to lactic acid by EMP pathway. Streptococcus diacetylactis produces diacetyls and acetoin that imparts nutty flavor and aroma to some sausage. *Staphylococci* also actively reduce nitrates to nitrites. Excessive nitrite level in sausage has been noted when using high nitrate concentrations with micrococci, producing a defect in sausage called “nitrite burn”. The micrococci are also lipolytic, and they produce lipase during early stages of fermentation. This results in an increase in free fatty acids, volatile fatty acids and carbonyl compounds after 28 days of drying. Lactic acid bacteria produce varying amounts of hydrogen peroxide, which is destroyed by the catalase of *micrococci*. Another important function of lactic acid bacteria is the inhibition of Staphylococci. This inhibition or suppression also suppresses the enterotoxin production by Staphylococci. This inhibition is more pronounced as the ratio of lactic acid bacteria to Staphylococci increases and as temperature of the fermentation decreases. The beneficial effects of lactic acid bacterial starter cultures in inhibiting Staphylococci and enterotoxin production in fermented sausages have also been demonstrated.

Sauerkraut

These are German terms for sour cabbage, which is generally prepared from shredded cabbage. The yellow -white shreds are approximately 2-5 mm in width and as long as 20 cm.

Standards for sauer kraut

It must contain at least 0.75% lactic acid and less than 10% of the total acid can be volatile. The pH must not exceed 4.1. The strainable brine should amount to about 10% of the total weight of sauerkraut and should contain from 0.7 to 3.0% NaCl.

Preparation for fermentation

Properly matured sound heads of cabbage are trimmed to remove the damaged parts and outer green or dirty

leaves. The cabbage is then sliced to shreds of size 0.16 to 0.08 cm in thickness. The shredded cabbage is conveyed to vats or tanks for salting and fermentation.

Role of salt

Salt plays a primary role in the preparation of sauerkraut; therefore its concentration is carefully controlled. According to legal standards salt must not be less than 2% and must not be more than 3%. Most of the producers of sauerkraut add salt in the concentration of 2.25 to 2.5%. Salt extracts water from shredded cabbage by the process of osmosis, thus forming fermentation brine. It suppresses the growth of some undesirable bacteria that might cause deterioration of the product and at the same time makes conditions favorable for the growth of lactic acid bacteria. Salt also contributes to the flavor of finished product by yielding a proper salt acid ratio. The use of too little salt causes softening of tissues and produces a product lacking in flavor. Too much fermentation and over salting may produce a product with a sharp, bitter taste. It may also cause darkening of the color of product and may favor the growth of pink yeasts. Brine begins to form once the shreds are salted and tank is closed when it is filled to the proper level. Then a weight is placed over these shreds so that it squeezes the water out of the shreds. The weight may be of wood (old method) or some plastic bag filled with water may be placed (modern method).

Microbiology of sauerkraut fermentation

Pederson first described the lactic acid bacteria that he observed in fermenting sauerkraut. He found that the fermentation was initiated by the species of Leuconostoc mesenteroides. This species was followed by gas forming rods and finally by non-gas forming rods and cocci. Leuconostoc mesenteroides is a heterofermentative bacteria and it grows more rapidly than other lactic acid bacteria. It is active over a wide range of temperature and salt concentrations. It produces acid and carbon dioxide that rapidly lowers the pH, thus inhibiting the activity of undesirable microorganisms and enzymes that may soften the shredded cabbage. The carbon dioxide replaces the air and creates an anaerobic condition favorable to prevent oxidation of ascorbic acid and natural color of the cabbage. It also stimulates the growth of many lactic acid bacteria. While this initial fermentation is developing, the heterofermentative species of lactobacillus brevis and homofermentative species of lactobacillus plantarum and sometimes Pediococcus cerevisiae begin to grow rapidly and contribute to the major end products like lactic acid, carbon dioxide, ethanol, acetic acid. Minor products also appear in the fermentation. The minor products are a variety of volatile compounds e.g. diacetyls, acetaldehyde and primary carbonyls.

Control of fermentation

Temperature, salt concentration and sanitary conditions are the primary environmental factors controlling the sauerkraut fermentation.

Influence of Temperature

At low temperature (7.5 °C), fermentation is very slow. Leuconostoc mesenteroides grows slowly attaining an acidity of 0.8-0.9% in terms of lactic acid in a month. Acidity is important for its preservative effect. Other lactobacilli and pediococci cannot grow at this low temperature. The sauerkraut may not be completely fermented for 6 months or more or until the temperature rises to a temperature suitable for the growth of higher lactic acid producing lactics. At a temperature of 18 °C with a salt concentration of 2.25%, a total acidity of 1.7-2.3% as lactic acid will be attained, with an acetic to lactic acid ratio of about 1:4 in about 20 days. At higher temperature i.e. 23 °C, the rate of fermentation will be greater so that a brine acidity of 1.0-1.5% (lactic acid) may be attained in 8 to 10 days. Active growth of Lactobacillus plantarum and Lactobacillus brevis may be initiated in 3-5 days and the kraut may be completely fermented in approximately one month. At still higher temperature of 32 °C, the rate of fermentation may be very rapid and an acidity of 1.8-2.0 may be attained in 8 to 10 days. The major share of the acid produced will result from the growth of homofermentative bacteria *L. plantarum* and *P. cerevisiae*. The flavor of the sauerkraut will be inferior, similar to an acidified cabbage. At the higher temperature sauerkraut will darken rapidly unless canned immediately. It will have a poorer shelf life than sauerkraut fermented at lower temperature. This sauerkraut also has low percentage of acetic acid and will not attain as high a total acidity, even though the pH is lower. It will also subject to yeast spoilage, partly because of its low content of carbon dioxide. It is also low in ascorbic acid content.

Influence of salt

Salt plays a primary role in the preparation of sauerkraut; therefore its concentration is carefully controlled. According to legal standards salt must not be less than 2% and must not be more than 3%. Most of the producers of sauerkraut add salt in the concentration of 2.25 to 2.5%. Salt extracts water from shredded cabbage by the process of osmosis, thus forming fermentation brine. It suppresses the growth of some undesirable bacteria that might cause deterioration of the product and at the same time makes conditions favorable for the growth of lactic acid bacteria. Salt also contributes to the flavor of finished product by yielding a proper salt acid ratio. The use of too little salt causes softening of tissues and produces a product lacking in flavor. Too much fermentation and over salting may produce a product with a sharp, bitter taste. It may also cause darkening of the color of product and may favor the growth of pink yeasts. Brine begins to form once the shreds are salted and tank is closed when it is filled to the proper level. Then a weight is placed over these shreds so that it squeezes the water out of the shreds. The weight may be of wood (old method) or some plastic bag filled with water may be placed (modern method).

Acetylcholine content in sauerkraut

Sauerkraut is known to provide certain laxative properties; both sauerkraut and its juice have been used as purgative. The strain of l. plantarum produces acetylcholine in the presence of choline, while simultaneously fermenting carbohydrates. This acetylcholine is of significance in nerve activity.

Defects and spoilage of sauerkraut

Pink-Kraut: Pink-kraut was observed first by Butjagin (1904) Wchmer (1905) and Henneberg

1916. Brunkow et. al (1925) and Fred and Peterson 1922 noted that this cause was the growth of pigmented yeast i.e. asporogenous yeasts presumably members of genus Rodotorula. Pederson and Kelly (1938) observed that Pink-kraut usually contained a salt content greater than 2.5%. They associated the growth of yeasts with any factor that would inhibit a normal fermentation or that would suppress or adversely affect the heterofermenting bacteria. Sometimes pink-kraut was observed in vats of sauerkraut only a few feet away from an area of soft-kraut. The latter condition arises due to insufficient salt concentration. Stamer (1975) reported that L. brevis produces a red pigment under certain condition that can be related to discoloration or darkening of sauerkraut. The red color occurs between pH 4.4 and 5.2 and is most readily generated under aerobic conditions. Chemical reducing agents like ascorbic acid, cystein or glutathione inhibit this color formation.

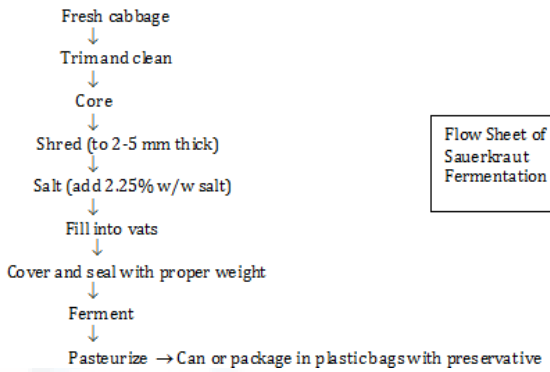
Slimy or Ropy Kraut: This is generally caused by dextran formation induced by the *L.mesenteroide* and is transitory in nature. This species prefers to ferment fructose rather than glucose, therefore in the fermentation of sucrose; the fructose is fermented leaving the glucose which interacts to form slimy, ropy water insoluble dextrans. These vary from an almost solid, gelatinous mass to ropy slime surrounding the bacterial cell.

Other Defects: Discoloration caused by autochemical oxidation. Loss of acidity caused bgrowth of molds and yeasts. Spoilage caused by molds and yeasts cause off-flavors and off-odors (yeasty and rancid). Slimy, softened kraut caused by aerobic growth of asporogenous yeasts.

Advantages of the acid food fermentations: 1) They render foods resistant to microbial spoilage and development of food toxins 2) They generally preserve the food between the time of harvest and consumption 3) they make the food less likely to transfer pathogenic microorganisms 4) they modify the flavor of the original ingredients and often improve the nutritional value. **(Flow Chart 10)**

Kimchi

Kimchi is the general name given to a group of fermented acid vegetable foods with a long tradition in Korea. More specific names are used for these pickled vegetables depending on the raw materials, processing methods, seasons and localities. Most kimchi is prepared at household level and consumed directly while limited amounts of cabbage-based kimchi are canned in factories and sold in the market. Kimchi is available throughout the year and, served three times a day, is a staple in the diet along with cooked rice and accessory side dishes. It is a favorite food unique in its complex of sour, sweet and hot pepper flavors accompanied by carbonation derived from fermentation with natural microflora. Kimchi differs from sauerkraut in two respects: 1) It has, optimally much less acid and 2) It is carbonated.



Flow Chart 10

Methods of preparation for typical korean kimchi (Tongbaechu-Kimchi or Kakduggi-kimchi)

Materials for kimchi preparation include 1) fresh vegetables (major vegetables are Korean cabbage, and radish; minor vegetables are garlic, green onion, ginger, leaf mustard, hot pepper, parsley pear, chest nut and carrot), 2) Jeotkal (Korean pickled fish) 3) fresh fish 4) seasoning agents (table salt, sesame seeds, sugar, monosodium glutamate, chenggak (type of seaweed), pear etc. additional minor ingredients may also be added depending on the household maker these are: saeujeot (pickled shrimp), meolchijeot (pickled anchovy), whangsegijeot, frozen Pollack, oyster, shrimp and small octopus. The ratio of major to minor ingredients varies depending upon the household maker; the range generally is 70-90 to 30-10. Although the proper combination of minor ingredients is reported to be the key to good-tasting kimchi, the most important factor seems to be the salt concentration. Salting of cabbage can be done at 5 to 7% for 12 hours or in 15% saline solution for 3-7 hours followed by rinsing and draining. Optimum salt concentration during kimchi fermentation is approximately 3% and is adjusted by experience at the household level. Fermentation of kimchi in the homes is usually carried out at ambient temperature. Using 3% salt concentration, the optimum fermentation period is one day at 30 °C, 2 to 3 days at 20 °C, 12-15 days at 10oC and 30-60 days at 5 °C. Optimum acidity of kimchi is 0.4 to 0.8% (as lactic acid). Higher acidity makes the product unacceptable.

Microorganisms

Kimchi is fermented by the microflora of the region. Organisms isolated include lactic acid bacteria, such as

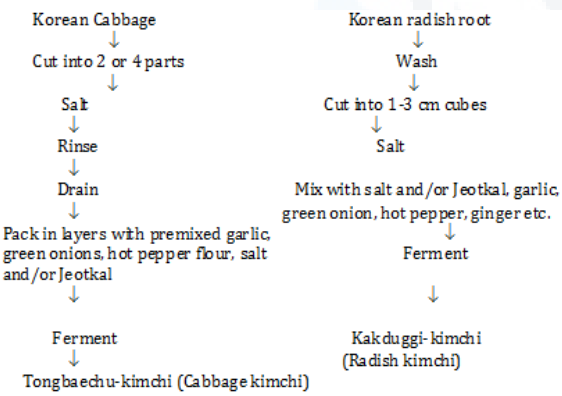
Leuconostoc mesenteroides, Streptococcus faecalis, Lactobacillus brevis, Pediococcus cerevisiae, *Lactobacillus plantarum*, and aerobic bacteria, such as *Achromobacter, flavobacterium and pseudomonas spp*. In the later stages of fermentation yeasts and molds appear that are reportedly causes of softening.

Preservation of kimchi

Kimchi is preserved at low temperature (below 5 °C) for a very short period of time. During storage at elevated temperature, rancidity and soft rot are accelerated by the microbial action. Thus shelf life is very short in summer months.

Biochemical changes in kimchi

The initial pH 5.5-5.8 falls to an optimum of 4.5-4.0. Optimum acidity (as lactic acid) is 0.4-0.8%. Salt concentration remains constant during fermentation. Kimchi fermented at low temperature (6-7 °C) contains more lactic, succinic, oxalic, tartaric, malonic, maleic, and glycolic acid than that fermented at 22-23 °C. Vitamins B_1, B_2, B_{12} and niacin reach the highest levels (twice the initial level) when kimchi possesses the most palatable taste and decrease when kimchi becomes sour. Vitamin C and carotene content decreases upon ripening. **(Flow Chart 11)**



Flow sheet of Tongbaechu-kimchi and Kakduggi-Kimchi

Flow Chart 11

Cucumber Pickles

The cucumber (Cucumis sativus) is popular both as a fresh and as a pickled vegetable. It is grown widely in temperate climates although originally of semitropical origin. Cucumbers for pickling must be grown from varieties known to have regular form, firm texture, and good pickling characteristics. Earlier varieties used for pickling were monoecious plants but new varieties developed by hybridization method have preponderance of female flowers and are called Gynoecious. These new cultivars often have greater vigor and uniformity than the open pollinated ones formerly grown. In addition, several of the hybrids are early maturing so they can be used to advantage in harvest scheduling. Pickling cucumbers are harvested while still immature. Fully grown (ripe) ones are undesirable for pickling because they are too large, change color and shape, are full of mature seeds, and are too soft for most commercial uses. After harvesting the cucumbers are immediately transferred to the salting station to avoid sweating i.e. growth of undesirable softening microorganisms. Unsound, decomposed, broken or crushed, distorted (wilt, rots, crooks, nubbins, etc.) are sorted out. They are then graded in a mechanical grader into 4 or more sizes. Three types of cucumber pickles are made: 1) Fresh pack -These are held in salt brine for as long as 2 days, then packed into jars or cans and pasteurized. They undergo marginal fermentation. 2) Salt Stock Pickles -From these pickles a variety of processed products are prepared. They undergo complete lactic acid fermentation. 3) Fermented Dill Pickles -They also undergo complete lactic acid fermentation. Dill herb is also added in these types of pickles.

Brining techniques for salt stock

There are two general methods for preparing salt stock pickles for fermentation these are Dry Salting and Brining

Dry Salting

For cucumbers, dry salting is done after first adding salt brine to cover the bottom of the tank (at least 12 in.) to form a cushion. This prevents bruising, breaking, or crushing the fresh cucumbers. Dry salt is then added at the rate of about 22.5 kg for every 450 kg of small cucumbers and 29.25 kg for every 450 kg large cucumbers. When full, the tank is covered with a wooden lid very tightly. Brine forms by osmosis. If the brine formed by osmosis does not cover cucumbers then 400 salometer brine is added to the desired level. The brine should be recirculated a day or two after tank is filled in order to equalize the concentration of salt throughout the brine. For long storage 60o salometer brine is used. In industry 100 salometer is equal to 2.64% NaCl by weight or 100o salometer is equal to 26.359 g NaCl at 15.5°C (saturated solution of salt).

Brine salting: Brine salting process is preferred over dry salting because dry salting yields soft,

flabby, shriveled pickles that do not fill out properly when processed. Therefore most picklers mostly use brine-salting technique for fermenting cucumbers. For brine salting 'low' or 'high' brine process may be used. In low brine salting, a salt brine of 25-30°C salometer is added into cucumbers whereas in high brine technique, 40°C salometer salt is added into cucumbers and tank is closed to air tight first by covering it with polythene sheet and then by lid. The cucumbers are handled by the same procedure as described in dry salting except brine is used to cover the cucumbers. Now a days, molded plastic and fiberglass tanks are being used in place of wood or concrete tanks. These plastic and fiberglass tanks have several advantages. These are 1) They are not subject to biological degradation or metal corrosion 2) They do not have to be maintained during the

off season, as do wooden tanks 3) As all the valves and piping are made of plastic, the problem of metal corrosion and contamination is eliminated 4) They are properly designed, closures are nearly airtight so problems of loss of acidity is reduced.

Microbiology of the cucumber fermentation

A rapid development of the microorganism causes a spontaneous fermentation as soon as the lid of the tank is closed after adding brine. The rapidity of fermentation is directly related to the temperature of the brine, concentration of the salt in brine, availability of fermenting materials and relative number of microorganisms available on cucumbers. Fresh cucumbers contain numerous and varied microflora including many potential spoilage microorganisms and a small number of lactic acid bacteria (5-103 acid forming bacteria per gram of cucumber). When cucumbers are brined at 5-8% NaCl range and allowed to undergo natural fermentation, the salt solution supports the fermentation by a sequence of various types of microorganisms. This sequence is categorized into four stages 1) Initiation 2) Primary fermentation 3) Secondary fermentation and 4) Post-fermentation

Initiation

This stage may include growth of many facultative and strictly anaerobic microorganisms originally present on the fresh material, the growth of undesirable microorganisms such as Gram-negative and spore-forming bacteria is inhibited as the pH gets lowered and lactic acid bacteria become established. The quality of the final product depends largely of the rapidity with which lactic acid bacteria are established and undesirable bacteria are excluded.

Primary fermentation

In this stage lactic acid bacteria (*Leuconostoc, Lactobacilli and Pediococci*) and both fermentative and oxidizing yeasts are the predominant active microflora. They grow in brine until the fermentable carbohydrates are exhausted or until there is production of lactic and acetic acids. In normal fermentation the undesirable microorganisms are excluded within 10-14 days. Buffering capacity and the fermentable carbohydrate content present in the medium are the important factors that govern the extent of fermentation by lactic acid bacteria and the extent of subsequent fermentation by yeasts.

Secondary fermentation

Pediococci, Lactobacillus brevis, and Lactobacillus plantarum and fermentative yeasts are responsible for the completion of lactic acid build up in this final stage of the fermentation. The acid tolerant yeasts still remain in the medium after the lactic acid bacteria are inhibited by low pH values and continue to grow till fermentable carbohydrates are exhausted.

Post fermentation

When fermentable carbohydrates are exhausted, microbial growth is restricted to the surface of brines exposed to air; the spoilage bacteria may become established on the surface of improperly managed tanks. At the end of fermentation, total acidity is 0.9% and pH is equal to 3.3.

Fermented dill pickles

Cucumbers when are subjected to bacterial fermentation in dill flavored, spiced, salt brine; the product obtained is called Dill pickles. They have their distinctive flavor and aroma due to the products of fermentation of lactic acid bacteria and to the blending of flavor and aroma of dill herb and spices that were added to the brine.

Method of preparation: The larger size cucumbers are washed and placed in suitable containers, together with the requisite amount of dill weed (which was earlier cured in vinegar, salt and brine) and dill spice and brine solution. Dill pickles are generally fermented in low salt (5% NaCl) brine solution. Vinegar is added to retard the growth of undesirable microorganisms (by decreasing the pH value). The fermentation is carried out at a temperature between 21 °C and 26.7 °C for 3-4 weeks. A curing period of 3-4 weeks further is also necessary. During this period, the flesh of pickles becomes entirely translucent and acidity about 0.5-1.2% (lactic acid). In addition, there is small amount of volatile acid (acetic acid); lactic acid bacteria and yeasts produce ethanol and other minor products.

Microbiology

In the beginning, a wide variety of unrelated microorganisms start growing in the fermentation but soon lactic acid bacteria predominate them. At low temperatures Leuconostoc mesenteroides play an important role in the fermentation. Once Leuconostoc starts predominating other species like Lactobacillus brevis, Lactobacillus plantarum begin to grow in the fermentation and ultimately complete the fermentation.

Packaging

Fermented dill pickles are marketed in bulk plastic containers and glass containers covered with acidified brine, closed and pasteurized at 74 °C for 15 minutes.

Spoilage of cucumber pickles

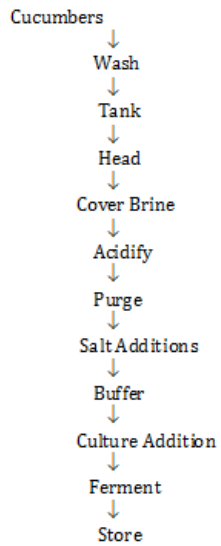
In Cucumber pickles, most of the deterioration is caused by the microorganisms; chemical defects are generally caused by metallic contamination, auto-chemical and physico-chemical reactions. Microorganisms damage the tissues by their cellulolytic or pectinolytic enzymes that result in loss of texture of firmness. Gaseous deterioration caused by microorganisms, resulting in the production of internal cavities or distorted stock caused by excessive gas pressure is another common spoilage. This defect is known as bloater or floater spoilage.

Softening of cucumber pickles

Softening occurs most frequently after brining of the cucumbers for dill or salt stock pickles. The entire skin of the cucumbers become slippery and can be removed easily. Such condition of pickles is sometimes referred to as 'Slip or Slippery pickles' in industry. When softening progresses into the deeper layers of cells and more and more pectic materials, present in the middle lamella separating the individual cells of the cucumber are attacked, the condition is known as 'Mushy Pickles'. A variety of bacteria of Gram-positive type (*Bacillus subtilis, B.pumilis, B.polymyxa, B.stearothermophilus*) and yeasts such as Saccharomyces fragilis, and Rhodotorula that produce pectinolytic and cellulolytic enzymes cause mushy deterioration.

Gaseous Spoilage of Cucumber Pickles

A number of genera of bacteria and yeasts cause gaseous spoilage in cucumber pickles. Undesirable yeasts produce gas because they utilize lactic acid and cause a rise in the pH. The fermenting yeasts have been identified as belonging to genera Brettanomyces, Hansenula, Saccharomyces and Torulopsis. Among bacteria, Lactobacillus brevis, Lactobacillus plantarum cause serious bloater spoilage. The major gases formed during spoilage are generally hydrogen and/or carbon dioxide. Nitrogen purging of the fermenting brine is used to reduce undesirable levels of carbon dioxide that otherwise might result in bloater formation. **(Flow Chart 12)**



Flow Sheet for Brine fermentation of Cucumbers.
Steps that have been added to the overall natural fermentation to render 'controlled fermentation' are indicated in bold face.

Flow Chart 12

Production of Industrial Enzymes

Introduction

Enzymes are biocatalysts produced within the living cells to bring about specific chemical changes. They are present in all living cells and the metabolic reactions common to all cells are catalyzed by these enzymes. The use of enzymes in industry dates back to some centuries before the discovery of enzymes. For example use of barley malt for starch conversion in brewing is a notable example. The field of enzymology was opened by Buchner brothers who showed that cell free extracts from yeasts fermented sugar to produce alcohol and carbon dioxide. In oriental countries microorganisms are directly employed as enzyme source for the preparation of products such as shoyu, miso, natto, and sake. Other important processes in which enzymes are primarily used are cheese making, leavening of bread, manufacture of vinegar, tanning of leather etc. It was Takamine who laid the foundation for the industrial production of microbial enzymes by developing process for producing diastase from fungi. Boidin and Effront of France were the first to produce industrial enzymes from bacteria. So far more than 1300 enzymes have been identified, out of which nearly 100 have been obtained in crystalline form.

Commercial production of industrial enzymes

Selection of microorganisms: The potential microorganisms are isolated from soil, decaying Organic matter or air and are tested individually for their capability to produce the desired product; this process is called primary screening. A few members of this potential natural isolates will possess the desired characteristics. It is also customary to grow the selected organisms on their substrates. In certain cases such as in the case of pectinases the organisms is induced to secrete the desired enzyme. The selected strains are maintained in pure form by lyophilization, on agar slant, or soil culture. The isolates are periodically checked for purity and for the retention of their original activity. Secondary screening is conducted in flasks or small fermentors. This evaluates the true potential of the organism to produce the desired product both qualitatively as well as quantitatively. Once potentiality of the organism is established, investigations are undertaken to work out a suitable medium and optimization of other conditions like pH, temperature, aeration etc. for the maximal enzyme production are carried out. Continuous maintenance of strains may cause degeneration and ability to produce the desired enzyme. Therefore, periodic re-isolation and reevaluation is necessary. The strains are also continually improved to enhance their capabilities by various physical agents such as UV treatment or by chemical methods. Recombinant DNA technology has also playean important role in the strain improvement programme.

Methods of cultivating microorganisms: Several methods of culturing the microorganisms are being employed industry that can be classified as follows: 1) Solid Culture -i) Conventional koji culture ii) Bulk koji culture iii) Rotary drum culture 2) Liquid culture -i) Stationary ii) Submerged These methods are explained in the coming section.

Preparation of starter culture: Working cultures are first prepared from stock culture (i.elyophilized or soil culture). These cultures are tested for their potency from time to time. In certain countries like Japan, there are firms that specialize in supplying pure fungal spores called Tane koji of desired strains of fungi to large manufacturing units. In such cases spores are directly inoculated into the growth medium. Starter inoculums for large fermentations (both for solid and liquid fermentations) have to be progressively built up. The quantity of inoculum required depends upon the batch size and it varies between 0.01 to 0.001 of the volume of the medium and is expressed as number of cells, weight of cell mass or just on the volume basis. Generally the amount of inoculum is kept as low as possible. Large Scale Mold Fermentation using Fungi

Bran process: Wheat bran is moistened with 0.2 to 0.3N HCl and autoclaved at 15lb pressure for1 hour. Addition of acid improves sterilization and inhibits growth of undesirable microorganisms. Sterilization can also be carried out by direct injection technique and continuously stirring the mass SO that bran particles come in direct contact with steam. When dilute acid is used for moistening bran, it is sufficient to hold the medium for 15-30 minutes in live steam to obtain practical sterility. The cooked bran is then cooled to room temperature and inoculated with inoculum grown earlier, at 1% level. It has been reported that 0.4% dry spore inoculum is sufficient for good growth of fungus. The inoculated bran is mixed well and transferred to trays having false bottoms. Layers of 5 centimeter are considered good for uniformly good growth. The trays are placed one above the other 8 to 10 cm apart. Spores germinate within 3-4 hours and temperature begins to rise after 5-6 hour. Aeration is started at this stage and continued till growth is completed. It takes 48-120 hours for the fermentation to complete depending upon the microorganism. After the completion of growth, the trays are shifted to drying tunnels with a central exhaust. The hot air is blown into the tunnel and air temperature is not allowed to rise above 40 °C. Different modification of the above mentioned process are adopted by different enzyme producing companies.

Bulk-koji process: In this method the fungus is cultivated on thick layers of bran up to 25cm t50cm high. The chamber has a false bottom and air is circulated under pressure from the bottom of the chamber. The chambers have a floor area of 2 to 30 meters by 6 to 10 meters. Temperature and humidity of the air in the chamber are automatically controlled.

Rotary drum method: In this method, the fermentation vessel consists of a rotating drum fitted with baffles so that the bran can be stirred. The chamber is also provided with cooling coils and an inlet for aeration. Bran is loaded into the drum and moistened with dilute acid and sterilized with steam. After cooling, the inoculum prepared in bran culture is added and mixed. After charging, the air is passed slowly into the drum which is maintained at a temperature of 28-30 °C. The spores germinate within 5-6 hours during which period drum is rotated slowly for 15-20 minutes after every 2 hours. The drum is rotated continuously for 5-6 hours at a speed of 1 rpm or less. The growth of the fungus is completed within 60 hours. The moldy bran is then spread in the form of layers on paper for drying. After drying, the moldy bran is ground and utilized as such, as an enzyme source or to extract the enzyme.

Extraction process: Powdered moldy bran prepared from the foregoing procedures is utilized for extraction and purification of the enzyme. The moldy mass is extracted with cold water (1-2 °C) or with solvents like ethanol. The common procedure is to employ counter current extraction system which gives a better and clear extract of enzyme. The quantity of water utilized generally is 5-10 times the weight of the moldy bran. The clear extract thus obtained is utilized for concentration and purification of the enzyme.

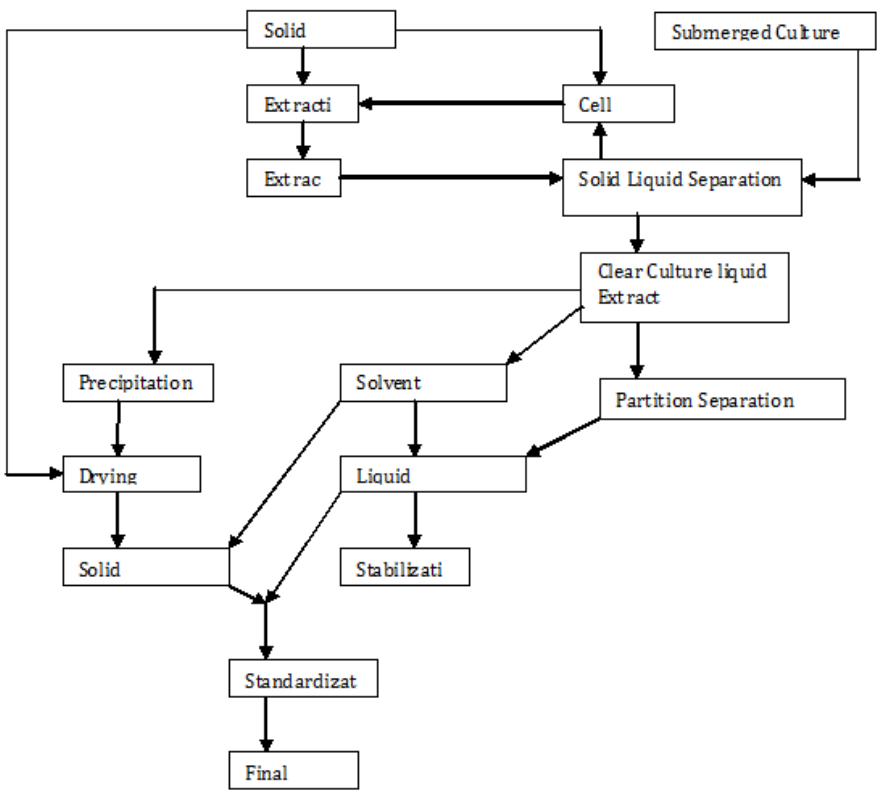
Submerged fermentation: After selecting a suitable microorganism capable of producing the desired enzyme and standardizing the conditions for its maximum output, large scale fermentation is taken up. This involves various operations that are discussed below: The substrate for growing microorganism is designed, based on the availability of cheap raw materials and types of enzyme to be produced. Composition of media recommended for different enzymes consists of components selected from starch hydrolysates, wheat bran extract, milled cereal products, soybean meal, peanut meal, corn steep liquor, distiller's solubles, yeast extracts and other organic and inorganic nitrogenous compounds and mineral salts. The liquid nutrient medium is charged into cleaned fermentors and sterilized by means of steam. The medium is constantly kept stirred during sterilization. The sterilization is carried out for 2-3 hours depending on the size of the batch. Generally the holding temperature is 121 °C for 20 minutes. For large fermentors a continuous high temperature and short time regime is adopted. Direct heating with steam is preferred when medium is thick and viscous. After sterilization medium is cooled to the desired temperature and pH is adjusted to optimum, the inoculum is introduced under aseptic conditions. In commercial production of microbial enzymes generally aerobic microorganisms are used for fermentation. In such cases aeration and agitation is started soon after inoculation. The fermentor is kept under constant pressure to avoid contamination. The agitator speed and quantity of air depends on the size of the batch, medium composition, and the requirements of the selected microorganism. After inoculation microorganisms establish themselves and passing through a lag phase, begin to grow exponentially. The standard fermentors are provided with aseptic sampling devices that allow samples

to be withdrawn for routine checking of contaminants, cell population, morphological observations and enzyme yield. Fermentation is carried out for 1-7 days. Most of the enzymes are secreted into the medium by the microorganism and extracellular enzymes appear in the medium during early part of the logarithmic phase. In case of intracellular enzymes cell disruption techniques are employed to recover the enzymes. The enzyme rich material obtained in any of the three procedures i.e. moldy bran, cultural extract, or cell autolysates is concentration and purified after solution is stabilized by the addition of chemical s such as ammonium phosphate, ascorbic acid, calcium salts, hydrochloric acid, phosphoric acid, sodium citrate, sodium phosphate, sodium sulphite or organic substances such as gelatin, gums, Arabic gum etc.

Purification of enzymes: The fermentation solution is subjected to centrifugation or filtration to obtain a clear liquid free of suspended particles. Concentration of the enzyme is brought by employing techniques such as vacuum evaporation and fractionation. In fractionation, all substances in the crude enzyme solution except the desired enzyme are separated out. Enzyme is then purified by precipitation, absorption, and crystallization. Precipitation of enzyme is carried out with salts such as ammonium sulphite, magnesium sulphite, sodium sulphite, or by other substances added to bring the pH of the solution to its isoelectric point to separate the protein. The former process is called as 'Salting Out' and latter referred to as 'Isoelectric Precipitation'. Water soluble solvents such as ethanol, methanol, isopropyl alcohol, acetone, dioxane etc. are also used for precipitating the enzyme. The precipitated enzyme is separated by process of centrifugation or filtration. The organic solvents are removed by low temperature drying and the inorganic salts by dialysis. Enzyme now gets concentrated manifolds. To obtain an enzyme in pure form, the enzyme solution is absorbed on ion exchange resins, inert earth, calcium phosphate, aluminum hydroxide, colloidal iron etc. after adjusting the pH to the optimum level. Highly purified enzyme is freeze-dried or crystallized.

Enzyme activity

Commercial enzymes are evaluated according to their specific activity per unit volume or weight. The activity is usually measured in 'Enzyme Units'. An enzyme unit indicates an amount of chemical change catalyzed by a definite quantity of enzyme. The activity can be standardized by blending the enzymes with inert materials such as diatomous earth, glucose, sucrose etc **(Flow Chart 13)**.



Flow Chart 13

Production of Vitamins

Riboflavin

Microbiologically produced Riboflavin has long been available in yeast and related preparations in association with many other vitamins of the B-complex category. Riboflavin is essential for the growth and reproduction of both humans and animals. Thus it is often incorporated into the feed of the animals. By fermentation process the riboflavin content of the medium can be raised up to 7 gm/L. Various microorganisms involved in the fermentation of Riboflavin are as follows: a) Ascomycetes- Ermothecium ashbyii and Ashbya gossypii are the commercial strains. b) Bacteria- recovered from the acetone butanol fermentation e.g. *Clostridium butylicum, C. acetobutylicum* and Mycobacterium smegmatis also produce riboflavin. c) Yeasts- *Candida guilliermondia, C. flareris* and Mycocandida riboflava are the non-commercial strains. (Figure 3)

Fermentation process for ascomycetes

Ascomycetes for the production of Riboflavin require semi purified sugars (glucose) and crude organic nutrients like corn steep liquor, animal stick liquor and meat scraps. Glucose may be totally replaced by corn oil, however low levels of corn oil may be added to the glucose to stimulate riboflavin yields. pH is adjusted to 6.5-7.5 and temperature 26-28 °C for 4-5 days. The fermentation is submerged, aerated but high levels of aeration may inhibit mycelial production and reduces the product yield. When Candida spp. are used for riboflavin production, the vessels made of steel cannot be used because the organism is very sensitive to traces of iron, therefore the vessels may be lined with plastic. Cobalt at proper concentration (stimulates the ascomycete fermentation) can be added into the fermentation broth as it partially counteracts the iron toxicity. Candida fermentation can be carried out at low pH, which eliminates the bacterial contamination and less sterilization is required.

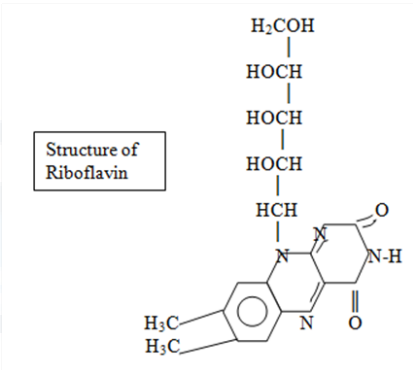
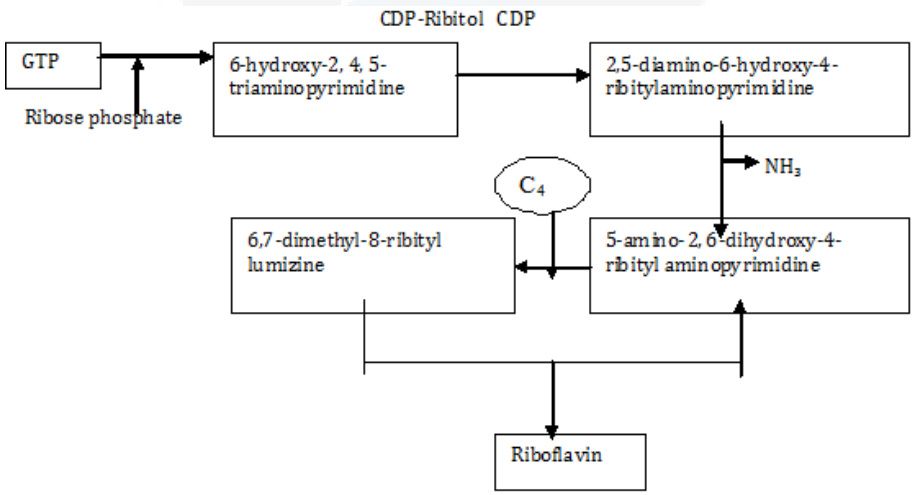


Figure 3

Mechanism of riboflavin accumulation

Kaprálek (1962) and Stárka (1957) demonstrated that fermentation of riboflavin progresses through three phases. First phase is a rapid growing phase with no riboflavin production. The substrate is utilized and oxidized; pH decreases as pyruvic acid accumulates. In the second phase glucose gets exhausted, sporulation begins, pyruvate decreases, ammonia accumulates because of deaminase activity, pH becomes alkaline, Rapid synthesis of cell bound riboflavin occurs in the form of FAD and rapid catalase activity causes disappearance of cytochromes. In the third phase autolysis of the cells occurs with the release of riboflavin into the medium **(Flow Chart 14)**.



Mechanism of Riboflavin Accumulation

Flow Chart 14

Recovery of riboflavin

On completion of fermentation, the solids were dried to a crude product for feed supplement. For a crystalline product, broth is heated for 1 hour at 15lb pressure to solubilize the riboflavin. Insoluble matter was removed by centrifugation and riboflavin is recovered by conversion to the less soluble form by chemical and microbiological methods. The precipitated riboflavin was then dissolved in water or polar solvents or in an alkaline solution, oxidized by aeration and recovered by recrystallization from aqueous or polar solvent solution or by acidification of the alkaline solution.

Carotenoid

Carotenes

Carotenes are precursors of vitamin A. Some carotenes are normally present in foods and have an essential biological function to perform. They are used as food supplement to prevent or cure vitamin deficiency diseases. In addition other pigmented carotenoids are used both as food additives for intensifying or modifying the color in fats, oils, cheese, and beverages and also as animal feed supplement to enhance the color of such foods as egg yolks and chicken flesh. Though carotenoids are widely found in plants and animals, only microorganisms and plants have the necessary systems to synthesize a wide range of these products.

Microorganisms

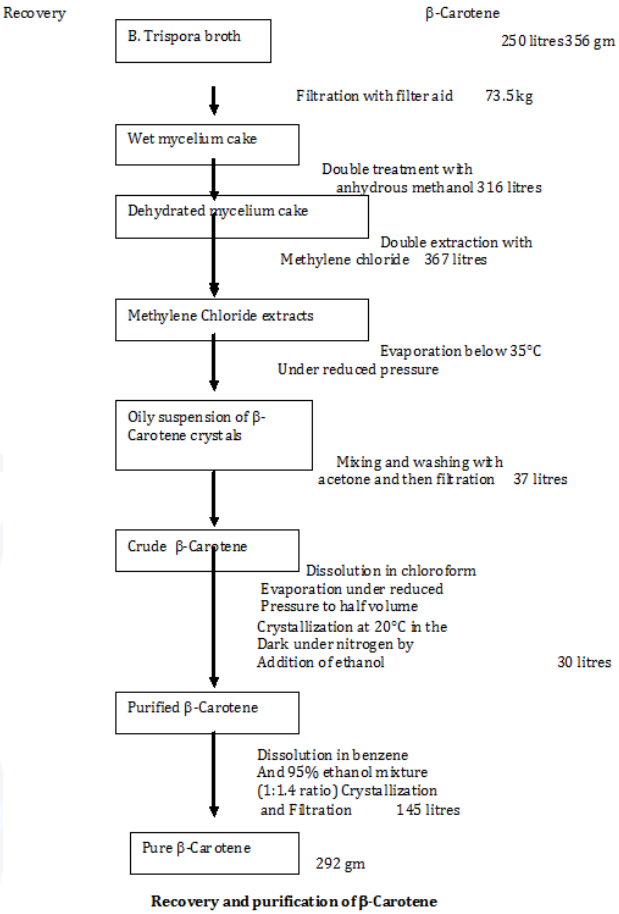
Many species of algae and fungi (e.g. *Neurospora crassa, Penicillium sclerotium, Phycomyces blakesleeanus*) and also yeasts (Rhodotorula) were considered for use in β-Carotene production but were found unsuitable. Some particular fungi in mucorales group and choanophoraceae family of Phycomyces concentrated the interest on the development of industrial fermentation. Particularly Blakeslea trispora have received extensive study for their ability to produce β-Carotene. This microorganism is heterothallic in nature. High concentrations of β-Carotene are produced only when both the mating types are present in the medium.

Growth conditions

The medium should be viscous and rich in vegetable oils, kerosene and surface-active agents, β or β- ionones are added during the incubation.

Activators of β-carotene production

β-Ionone, a precursor of β-Carotene is not directly incorporated into the β-Carotene but it activates the enzymes required for carotene production. β-Carotene production can also be enhanced by the presence of dimethyl formamide, α-pyrrolidone and succinimide in addition to β-ionones. **(Flow Chart 15)**



Recovery and purification of β-Carotene

Flow Chart 15

Recovery and Purification of β-Carotene

Crystallization

It is the formation of solid particles within a homogenous phase. It may occur as the formation of solid particles in a vapor, as solidification from a liquid melt or as crystallization from liquid solution. Crystallization from solution is important industrially because of the variety of materials that are marketed are in crystalline form. In industrial crystallization from solution, the two-phase mixture of mother liquor and crystals of cell sizes that occupy the crystallizer and withdrawn as product is called Magma. Crystals have been classified into seven classes these are: cubic, hexagonal, trigonal, tetragonal, orthorhombic, monoclinic, and triclinic. A given material may crystallize in two or more different classes depending upon the conditions or crystallization e.g. calcium carbonate occur commonly in nature in hexagonal form (as calcite) but also it occurs in the orthorhombic form (as aragonite). Under ideal conditions, a growing crystal maintains geometric similarity during growth; such a crystal is called invariant. Unless the crystal is a regular polyhedron,

the rates of growth of various faces of an invariant crystal are not equal.

Principles of crystallization

Crystallization may be analyzed from the standpoint of purity, yield, energy requirements, rates of nucleation and growth. Purity- crystals are purified from the mother liquor by filtration, centrifugation and then washing the crystals with fresh solvent. The effectiveness of these purification steps depends upon the size and uniformity of the crystals. Equilibrium- Equilibrium in crystallization process is reached when the solution is saturated and equilibrium relationship for bulk crystals is the solubility curve. Yields- In many industrial crystallization processes, the crystals and mother liquor are in contact long enough to reach equilibrium, and the mother liquor is saturated at the final temperature of the process. The yield of the process can then be calculated from the concentration of the original solution and the solubility at the final temperature. Supersaturation- In the formation of a crystal two steps are required 1) the birth of a new particle 2) its growth to macroscopic size. The first step is called Nucleation.

Types of crystallizers

Commercial Crystallizers are different in several ways. The difference lies in how the crystals are brought into contact with supersaturated liquid. The first technique called the 'Circulated liquid method', a stream of supersaturated solution is passed through a fluidized bed of growing crystals, within which supersaturation is released by nucleation and growth. The saturated liquid then is pumped through a cooling or evaporating zone, in which supersaturation is generated and finally the supersaturated solution is recycled through crystallizing zone. In the second technique called 'Circulating Magma method', the entire magma is circulated through both crystallization and supersaturation steps without separating the liquid from solid. Supersaturation as well as crystallization occurs in the presence of crystals. In both the methods feed solution is added to the circulating stream between crystallizing and supersaturating zone.

Precipitation

Precipitation phenomenon is used to obtain products from the broth or some times, to remove impurities from the ongoing fermentation process. There are a number of processes where insoluble precipitates are isolated. Since organic solutes have solubilities dependent on the solution temperature, pH, composition, ionic strength and dielectric constant therefore precipitation can be brought about in many ways as follows:

1) By adding precipitant to react with solute and producing an insoluble product, often a salt e.g. procaine hydrochloride + penicillin β Procaine-penicillin.

Organic solvent + streptomycin + sulfuric acid β Dihydrostreptomycin sulfate

Organic solvent + Erythromycin + water β Erythromycin hydrate

Biopolymer recovery is also obtainable by salt addition e.g. Xanthan gum is a polyanion and calcium ion can be used to form gel precipitate. Alginate biopolymer is recoverable from algal biomass by cell removal (filtration), followed by calcium chloride precipitation of the biopolymer.

2) Solvent driven precipitations are useful in the production of microbial biopolysaccharides including dextrans and xanthan gums. The biogum fermentations are typically aerobic and produce a highly viscous final broth with xanthan production, final broth pasteurization kills Xanthomonas cells. After adding KCl and then methanol or isopropyl alcohol the gum polysaccharide directly precipitates out. Dextran recovery is achieved by alcohol or acetone precipitation. In solvent driven precipitation for the production of bulk polysaccharide, the modest product value requires efficient recovery and reuse of solvent as well as good solvent removal for food or pharmaceutical grade product. 3) Protein precipitation techniques- The techniques result in a phase change to form a precipitate, require some alteration of protein solution conditions to render the original, thermodynamically stable one phase system unstable with respect to precipitation. The various methods for causing the needed reduction in solubility of protein include: 1) Added high salt concentration to give precipitates by salting out 2) pH adjustments to protein's pH of neutral charge, the isoelectric point, at which point the protein has minimum solubility 3) Reduction of medium dielectric constant to enhance electrostatic interaction by e.g. addition of miscible organic solvent 4) Addition of non-ionic polymers that reduce the amount of water available for protein solvation 5) Addition of polyvalent metal ion to form reversibly a protein precipitate. The method of choice includes considerations not only of the protein concentration needed and cost of separation technique, but also the purity of the final product compared to precipitating agent **(Table 2)**.

Recovery and purification of microbial products

When biosynthesis of products in a fermentor takes place this becomes necessary to isolate the product and convert it into a form suitable for the required purpose. The isolation procedures differ considerably depending upon the location of the product i.e. intracellular or extracellular and also depend upon the concentration and stability of the product. Sometimes microorganisms also produce many other organic products apart from the main product that may complicate the process of isolation. The simplest situation is the isolation of microbial cells when they represent the desired end product. The basic process to isolate the microbial products is shown in the following figure: **(Flow Chart 15)**

After cultivation, the culture fluid is usually processed in order to facilitate the separation of microorganisms. The treatment depends upon the composition of the fluid, the

type of cultured microorganisms and the product; it may include an adjustment of pH, heating and /or addition of substances that coagulate the microbial cells. After such treatments the microorganisms are separated by filtration or centrifugation. Further treatments depend on whether the product is intracellular or extracellular in the supernatant. The microorganisms themselves, or the filtrate, may, after suitable processing by pressing, evaporation, and etc. constitute the end product. If the product is contained in the cells, it is necessary first to disintegrate them; the method of disintegration again depends upon the type of microorganism, physiological state and composition of the cell wall. Following disintegration, the cell walls are separated and product is isolated by the methods shown in the above figure. Products contained in the supernatant are isolated-depending on their chemical properties-by precipitation, extraction, adsorption, dialysis, ultrafiltration, evaporation etc.; these methods are often used in combination. After isolation, the products are further processed to a form they are to be used for the said purpose (in medicine, food industry, agriculture, etc.) **(Table 3)**

Table 3

Parameter	Grape Juice (mls)	Fermenting Wine		
		7 days	14 days	21 days
% Tartaric acid				
% Acetic acid				
Alcohol				
Taste				
Aroma				
Clarity				

Mechanical separation of microorganisms

The choice of the method of mechanical separation and the appropriate equipment depend on the type of microorganism (Bacteria, yeast, actinomycetes or filamentous fungi), composition of the medium (synthetic or complex) and the absence or presence of suspended particles. Basically, the separation of microorganisms is carried out using one or two main methods, filtration and centrifugation.

Filtration

It may be defined as the separation of suspended particles from a liquid by means of a pressure difference through a permeable partition. The diameters of the particles may be smaller than the openings in the filter so that initially they pass through these openings. When, however, the filter pores become clogged with the particles, the filter begins to retain further particles almost completely. As filtration proceeds the thickness of the particle cake on the filter increases and the flow rate of the filtrate decreases. The gradual decrease in flow rate is caused partly by clogging of the pores on the filter surface and partly also by the diminishing distance between particles inside the pores. For rigid or non-compressible particles the filter cake may be considered as a system comprising a large number of capillary channels through which the fluid flows according to Poiseuille's law. Then

$$u = \frac{dV}{A\,dt} = \frac{K'\,\Delta P}{\mu L}$$

Where u = linear flow rate; V= filtrate volume; T= time; P= pressure drop through filter cake; L= thickness of filter cake; A= filter area; and = viscosity of fluid

$$\frac{dV}{dt} = \frac{K'A\,\Delta P}{\mu L}$$

$$\alpha = \frac{1}{K'} = \frac{A\,\Delta P}{\mu L} \div \frac{dV}{dt}$$

Food Fermentation Practical (1) -To study the wine production by the fermentative activity of Yeast cells.

I. **Principle**

Wine is a product of the natural fermentation of the juices of grapes and other fruits such as peaches, pears, plums, and apples by the action of yeast cells. This biochemical conversion of juice to wine occurs when the yeast cells enzymatically degrade the fruit sugars, fructose and glucose firstly into acetaldehyde and then into alcohol. Grapes containing 20-30% sugar contents will yield wines with an alcohol content of 10-15%. Also present in grapes are acids and minerals whose concentration are increased in the finished product and are responsible for the characteristic taste and bouquet of different wines. For red wines crushed grapes must be fermented with their skins to allow extraction of their color into juices while white wines are produced from the juice of white grapes without skins. The commercial production of wine is a long and exacting process. First the grapes are crushed to express juice called "must". Potassium metabisulfite is added to the must to retard the growth of acetic acid bacteria, molds, and wild yeasts that are endogenous to grapes in the vineyard. A wine producing yeast Saccharomyces cerevisiae var ellipsoideus is used to inoculate the must that is used to inoculate the must, which is then incubated for 3-5 days under aerobic conditions at 21-32oC. This is followed by an anaerobic incubation period. The wine is then aged for a period of one to 5 years in aging tanks or wooden barrels. During this time the wine is clarified of any turbidity and formation of esters responsible for characteristic flavor are produced. The clarified product is then filtered, pasteurized at 60oC for 30 minutes and bottled.

II. **Materials required**

Fifty mls of white grape juice broth culture of Saccharomyces cerevisiae incubated for 48 hours at 25oC. Five hundred mls of pasteurized Welch commercial white grape juice. Phenolphthalein solution 1%. Sodium hydroxide 0.1N and sucrose. One litre Erlenmeyer flask, One holed rubber stopper containing a 2" glass tube plugged with cotton plug, Pan balance, Spatula, Glassine paper, 10 ml graduated cylinder, Ebulliometer, Burette and Pipettes for titration.

III. **Procedure**

Pour 500 ml of white grape juice into one litre Erlenmeyer flask. Add 20gm sucrose and 50 ml of Saccharomyces cerevisiae containing grape juice broth culture (10% starting culture). Close the flask with the stopper containing cotton plugged air vent. Incubate the wine at 25 °C. After 2nd and 4th day of incubation, add 20gm more sucrose to the fermenting wine. Now again incubate at 25oC for 21 days.

IV. **Total acidity (expressed as % Tartaric acid)**

To 10 ml of aliquot of fermenting wine, add 10ml distilled water and 5 drops of phenolphthalein indicator. Mix and titrate it with 0.1N NaOH solution. (Table 2)

$$\% \text{ Tartaric acid} = \frac{\text{Mls of Alkali} \times \text{Normality of Alkali} \times 7.5}{\text{Weight of sample (1 ml of wine = 1gm of wine)}}$$

Volatile Acidity (expressed as %Acetic acid)

$$\% \text{ Acetic acid} = \frac{\text{Mls of Alkali} \times \text{Normality of Alkali} \times 6.0}{\text{Weight of sample in grams (1ml of wine = 1gm of wine)}}$$

Theory

Milk of high SNF (10-12 or 15%) is used for the preparation of yogurt. It is different from curd in the sense that, in yogurt milk of high SNF is inoculated with pure culture of *Lactobacillus bulgaricus and Streptococcus cremoris or Streptococcus thermophilus* in the ratio of 1:1 while in curds natural flora acts as inoculum. After fermentation, the acidity of the final product is measured in terms of lactic acid (gm per 100ml of yogurt).

V. **Procedure**

Preparation of starter culture: The inoculum of Lactobacillus bulgaricus is maintained in Micro-inoculum broth of composition: Yeast extract 20g, Peptone 5g, Dextrose 10g, Potassium dihydrogen phosphate 2g, Sorbitan monooleate complex 0.1g or Tween-80 few drops, and distilled water one litre. The inoculum of Streptococci is maintained in Nutrient broth of composition: Beef extract 3g, Peptone 5g, NaCl 5g, and distilled water one litre. Cultures of both the strains are to be mixed in 1:1 ratio in Peptonized milk of composition: Skim milk powder 10g, Peptone 5g, and distilled water 100 ml; pH 6.5 sterilized at 85-95oC for 40-60 minutes; and incubated at 21-28oC for 12-18 hours till the pH reaches 4.9-5.0. The starter culture now is cooled to 5-10 °C and is kept at the same temperature until used. It is not a good idea to hold ripe starter for more than 24 hours.

VI. **Fermentation**

Weigh desired quantity of milk and adjust its SNF to 10-12% by adding whole dry milk. Add sugar at the rate of 10%. Heat it to 80-90 °C for 20 minutes. Cool the milk to 45-48 °C (for S. thermophilus) and inoculate with 5% yogurt starter culture. Mix well. Keep the milk in clean and sterile container for setting. Incubate the milk containers at 45 °C for 3-4 hours till a firm coagulum is obtained. Remove the product from incubator and keep it at 5 °C till it is consumed.

Calculation of Milk Solid Non Fat (MSNF)

Milk at a temperature of 60oF is added up to the brim of the cylinder and lactometer is gently dropped into it. Reading on the lactometer is noted down. This reading is corrected as follows:

- ✓ Add one for every 10oF rise in temperature
- ✓ Add 0.5 for upper meniscus of the milk

This now is called as Corrected lactometer reading (CLR). The MSNF is calculated by the formula as: MSNF = CLR/4 + 0.2 × Fat + 0.14

E.g. If lactometer reading observed at 70oF comes out to be 27 then CLR is equal to 27+1+0.5 (for meniscus) = 28.5 and MSNF = 28.5/4 + 0.2× Fat + 0.14

Calculation of Acidity in Yogurt

Take one gram of yogurt in titration flask and dilute it with 5 ml of distilled water. Add to it a few drops of phenolphthalein indicator. The solution is colorless. Now add from the burette 0.1N NaOH solution drop wise till the color of solution changes to light pink. This is the end point. Repeat the experiment till a concordant set of three readings is obtained.

General Calculations

Take one gram of yogurt in titration flask and dilute it with 5 ml of distilled water. Add to it a few drops of phenolphthalein indicator. The solution is colorless. Now add from the burette 0.1N NaOH solution drop wise till the color of solution changes to light pink. This is the end point. Repeat the experiment till a concordant set of three readings is obtained.

VII. **Theory**

Natural microflora present on cabbage produces lactic acid from carbohydrates. In due course of time, after the accumulation of lactic acid to certain extent, all proteolytic and other microorganisms are eliminated from the product except lactic acid tolerant Lactobacilli. These bacteria further produce more lactic acid resulting in lowering of the pH of the product significantly after 3-4 weeks. Because of this lowering in pH other organisms do not find any access to grow in the same product.

$$\% \text{ Acidity} = \frac{\text{ML of Alkali used} \times \text{Normality of Alkali} \times 0.09}{\text{Volume of sample}}$$

(In terms of Lactic acid gm/ml)

0.09 is gram equivalence of lactic acid per ml.

Production Chart of Yogurt

Quantity of milk used _____kg Quality of fresh/ old/ pasteurized milk _____

Fat _____% MSNF_____% Acidity of milk_____%

Temperature of Heating ___ Quantity of milk used _____kg Quality of fresh/ old/ pasteurized milk _____

Fat _____% MSNF_____% Acidity of milk_____%

Temperature of Heating ___°C Temperature of Holding ___°C Temperature of Cooling____°C

Starter used_____Quantity of starter used_____

Texture_____Time of incubation_____ Quality of Yogurt_____Good/fair /Poor

Acidity of Yogurt _____gm/100ml Temperature of Storage_____°C.

Temperature of Holding ___°C Temperature of Cooling____°C

Starter used_____Quantity of starter used_____

Texture_____Time of incubation_____ Quality of Yogurt_____Good/fair /Poor

Acidity of Yogurt _____gm/100ml Temperature of Storage_____ Quantity of milk used _____kg

Quality of fresh/ old/ pasteurized milk _____

Fat _____% MSNF_____% Acidity of milk_____%

Temperature of Heating ___°C Temperature of Holding ___°C Temperature of Cooling____°C

Starter used_____Quantity of starter used_____

Texture_____Time of incubation_____ Quality of Yogurt_____Good/fair /Poor

Acidity of Yogurt _____gm/100ml Temperature of Storage_____°C.

VIII. **Procedure**

Wash cabbage with clean water. Remove the outer leaves. These leaves are kept aside for their further use. Remove the case and other undesirable area. Prepare lots of cabbage weighing ½ kg each and slice them into shredding or small pieces of 0.16-0.08 cm in thickness. Weigh the salt (2.25%) i.e. 11.25 gm for ½ kg of cabbage shredding. Put the outer leaves that were kept aside, at the bottom of a glass jar. Take one lot of shredded cabbage and layer onto the leaves inside the glass jar. Sprinkle ¼th of the salt on the cabbage and wait for its absorption in the shredded cabbage. Similarly layer the rest of the shredded

cabbage and sprinkle the salt onto it till all the cabbage and salt finishes for the preparation of sauerkraut. The addition of salt serves two main functions. Firstly, it draws moisture out of cabbage that dissolves the salt forming a brine solution, which acts as a fermenting medium for Lactobacilli and also equally distributes them in the medium. Secondly, it inhibits the growth of proteolytic bacteria. Now place the plastic bags filled with water as a weight to press the cabbage and close the lid of the glass jar. Fermentation was carried out at 25oC for 3-4 weeks. Note down the change in pH and color after every week.

Microbiology of Sauerkraut Fermentation Pederson first described the lactic acid bacteria that he observed in fermenting sauerkraut. He found that the fermentation was initiated by the species of Leuconostoc mesenteroides. This species was followed by gas forming rods and finally by non-gas forming rods and cocci. Leuconostoc mesenteroides is a hetero fermentative bacteria and it grows more rapidly than other lactic acid bacteria. It is active over a wide range of temperature and salt concentrations. It produces acid and carbon dioxide that rapidly lowers the pH, thus inhibiting the activity of undesirable microorganisms and enzymes that may soften the shredded cabbage. The carbon dioxide replaces the air and creates an anaerobic condition favorable to prevent oxidation of ascorbic acid and natural color of the cabbage. It also stimulates the growth of many lactic acid bacteria. While this initial fermentation is developing, the hetero fermentative species of lactobacillus brevis and homofermentative species of lactobacillus plantarum and sometimes Pediococcus cerevisiae begin to grow rapidly and contribute to the major end products like lactic acid, carbon dioxide, ethanol, acetic acid. Minor products also appear in the fermentation. The minor products are a variety of volatile compounds e.g. diacetyls, acetaldehyde and primary carbonyls.

Role of temperature in sauerkraut fermentation

Lower the initial temperature better is the product formation. It is considered that the initial temperature of 18.3oC produces superior quality sauerkraut because at lower temperature hetero fermentative lactic acid bacteria exert a greater effect.

Spoilage of Sauerkraut

Common spoilage signs found in sauerkraut are discoloration, off-flavor, off-odor caused by yeast and mold growth, loss of acidity and slimy product due to the dextran formation by Leuconostoc mesenteroides. The proteolytic activity of molds and yeasts and also by asporogenous yeasts produces product pink in color. Such type of spoilage is known as “Pink kraut”.

Food Fermentation practical (4) -Preparation of Sweet Acidophilus milk

I. **Theory**

The fermented acidophilus milk is known for its therapeutic value and has been successfully tried in cases of chronic colitis and gastro-intestinal disorders in general. It is prepared by the inoculation of pure culture of Lactobacillus acidophilus. It is a very acidic product. The acidophilus milk has not gained popularity as that of other fermented milk products because of its taste and off-flavor. When it is sweetened with sugar, it is called as sweet acidophilus milk. Sweet acidophilus milk is gaining popularity now a day.

Medium for maintenance for lactobacillus acidophilus

The organism is maintained in Tomato Juice Agar of Composition: Tomato juice 400ml equivalent to 20g tomatoes, Peptone 15g, Skimmed milk powder 10g, Agar-Agar 1.5-2.0%; pH 5.0; sterilize at 15lb pressure for 15 minutes.

Procedure for ordinary acidophilus milk

Take adequate quantity of low fat milk. Boil or steam it for 20 minutes. Cool it to a temperature of 28-30^{0}C. Pass carbon dioxide gas through it for 1-2 minutes. Inoculate it with 1-2% inoculum of Lactobacillus acidophilus. Incubate at 37-40 °C for 30-40 hours.

Procedure for sweetened acidophilus milk

Inoculate cold pasteurized sweetened low fat milk with 1-2% of pure culture of Lactobacillus acidophilus. No incubation is done. Inoculated milk is held under refrigeration at 7°C or below for 30-40 hours or till it is consumed. It tastes exactly like low fat milk.

Food Fermentation Practical (5) -Production of Lactic acid

Materials required

Pure culture of Lactobacillus delbrueckii B-70 and Production medium of composition: Sucrose 100g/L, Yeast extract 20g/L, Potassium dihydrogen orthophosphate 2.5g/L, Calcium carbonate 10%, Agar-Agar 2% (for solid medium); pH 7.0-7.2; Sterilize at 15 lb pressure for 15 minutes. Vitamin B-complex+ Aspartic acid +Folic acid combination may be used in place of yeast extract.

Medium for inoculum preparation

Inoculum is prepared in Glucose Yeast Extract medium of composition: Glucose 10%, Yeast extract 2% and Potassium dihydrogen orthophosphate 0.25%.

I. **Procedure**

Take two litres of production medium in laboratory fermenter and inoculate it with 10% of inoculum of L. delbrueckii B-70 prepared in Glucose Yeast extract medium. Incubate it at 37 °C for 5-7 days. The medium is gently stirred during the incubation period to keep the calcium carbonate in suspension.

Recovery of lactic acid

Filtration -The suspension is filtered with conventional laboratory filters. Acidification -To the filtrate add concentrated sulfuric acid to form precipitates of calcium sulfate. Filter and wash the precipitates with water. The washings are added into the filtrate. Removal of Impurities -The filtrate is treated with activated charcoal to remove the impurities. Filter again. Concentration –The filtrate is evaporated on a steam bath to concentrate it to 25% solids and then again subjected to evaporation till 50% solids are obtained. Removal of Heavy metals -The heavy metals like lead is removed by adding sodium or potassium ferrocyanide into the concentrated mass and filtering it. The filtrate contains lactic acid, which can be purified by passing it through ion exchange resin column. The lactic acid so obtained may have 93-95% purity.

Food Fermentation Practical (6) –Production of Amylase enzyme and its estimation

Materials required

Inoculum -An Amylase producing strain of Streptomyces spp. preserved in Nutrient medium of composition: Beef extract 3g, Peptone 5g, NaCl 5g, and distilled water one litre. Calcium chloride solution 1% in water, Starch solution 0.1% in 0.05M acetate buffer pH 5.2 (dissolve 1mg starch in 1ml of acetate buffer), Production medium of composition: Beef extract 3g, Peptone 5g, NaCl 5g, and distilled water one litre. Add starch 10g per litre into the nutrient broth medium. Sodium acetate-Acetic acid buffer solution (0.05M) -Sodium acetate 2.72g dissolve in 100ml distilled water (solution-I), Acetic acid 1.15g dissolve in 100ml distilled water (solution-II); add solution-I into solution-II and adjust pH to 5.2, make volume 100ml and heat it to 60oC for 10-15 minutes.

I. Procedure

Dispense 100ml of production medium in two 250ml conical flasks and sterilize it at 15lb pressure for 15 minutes. Inoculate the flasks with Streptomyces spp. culture that was preserved in nutrient broth medium at the rate of 5%. Incubate the flaks on shaker at 37 °C for 96 hours.

Standard curve for starch

Prepare dilutions of starch in acetate buffer from 0.1% to 0.01%. To each of these dilutions, add 0.2ml Iodine solution and add water to dilute it to 10ml. Measure the optical density at 520nm. For control take 1ml of distilled water in place of starch dilution and to this add iodine solution; observe optical density in a similar manner.

Estimation of amylase

Prepare Control, Blank and Digest. Control -To 6ml of 0.1% starch solution, add 1ml of 1% calcium chloride, 2ml of hydrochloric acid, and 1ml of distilled water and 0.2ml of iodine solution. Measure optical density at 520nm. Blank -To 6ml of 0.1% starch solution, add 1ml of 1% calcium chloride solution, 1ml of enzyme extract, 2ml of hydrochloric acid and 2ml of iodine solution. Measure optical density at 520nm. Digest -To 6ml of 0.1% starch solution, add 1ml of 1% calcium chloride solution and 1ml of enzyme extract. This is incubated at 30 °C for 10 minutes. Now add 2ml of HCl and 0.2ml of iodine solution. Measure optical density at 52 nm.

Calculation of Enzyme activity

$$\text{Enzyme Activity (mg/minute or IU/ml)} = \frac{\text{1mg/ml Starch} \times (\text{OD Control} - \text{OD Digest})}{\text{Time of Incubation}}$$

Food Fermentation practical (7) – To study the alcoholic fermentation by Yeast

I. Theory

The yeast Saccharomyces cerevisiae converts fermentable sugars (glucose, fructose and sucrose) into ethanol and carbon dioxide. In large-scale production of alcohol, molasses is used as substrate. Blackstrap molasses contains 45-55% w/v fermentable sugar as sucrose, which is metabolized by yeast through Embden-Meyerhoff-Parnas Pathway to produce ethanol and carbon dioxide as the end products.

Materials required

Molasses; fermentation jar (10 L); micro distillation unit; Test tubes; conical flasks; Standard volumetric flasks (25ml); Urea; DNS reagent (3,5-Dinitosalicylic acid 1%, Phenol 0.2%, Sodium carbonate 0.05%, Sodium hydroxide 1% and Sodium potassium tartarate 20%); Dichromate reagent (Dissolve 34 gm of Potassium dichromate in 500 ml of distilled water, add 325 ml of concentrated Sulfuric acid slowly keeping the flask in ice bucket); YPD medium of composition: Yeast extract 10gm, Peptone 20 gm,

Dextrose 20gm and Agar-Agar 20gm; Slant culture of yeast S. cerevisiae.

I. Procedure

Preparation of Inoculum: Prepare 250ml GYE / YPD broth medium. Add 50ml in separate flasks and autoclave at 15lb pressure for 15 min. Inoculate slant culture of yeast aseptically in each 50ml flask. Incubate the flasks at 28 °C for 16-18h.

II. Inoculum for fermentation

Dilute molasses to 12 ^{o}Brix (1.1Kg molasses in 8 litre of water). Adjust pH to 5.5 with 10N Sulphuric acid. Sterilize at 10lb pressure for 30min and then cool. Inoculate this 250 ml molasses medium (2 flasks) with 10% of the culture grown in YPD. Incubate the culture in shaker for 12h at 28 °C.

III. Fermentation medium

Dilute the Black strap molasses with tap water to 22 ^{0}Brix (2.1Kg molasses in 8 litre water). Adjust pH to 5.5 with 10N Sulphuric acid. Add 200mg Sodium dihydrogen phosphate and 200mg Urea per litre respectively. Maintain pH 5.5. Sterilize medium at 10lb pressure for 30min. Inoculate with 10% v/v inoculum and incubate at 28 °C for 48-72h for fermentation. After the fermentation has ceased close the mouth of the flask with an airtight bung to provide anaerobic conditions so that alcohol production may take place. Withdraw 10ml sample at every 12h interval and estimate alcohol and sugar concentration. Plot a graph with time on X axis and alcohol and sugar concentration on Y- axis.

Alcohol estimation

Preparation of standard curve for alcohol concentration: Prepare 1-10% v/v alcohol in

Various test tubes. Take 1ml of the various concentration of alcohol into 25ml of distilled water in 100ml distillation flask fitted with Liebig condenser. Distill and collect 15ml of the distillate in a 50ml volumetric flask containing 25ml Pot. Dichromate solution. Make up the volume to 50ml. Keep the alcohol-Pot. dichromate complex at 60 °C for 30 min. Measure OD at 600nm. Plot a standard curve with concentration of alcohol on X-axis and OD at 600nm on Y-axis.

Determination of Alcohol Concentration from fermentation Medium

Take 1ml sample and mix it with 25ml of distilled water. Distill as above and measure OD at 600nm. Find the alcohol concentration from the standard graph.

A. If molasses could not be obtained for laboratory exercise, use YPD medium with 50g/L sucrose for inoculum development and with 150g/L sucrose for preparation of fermentation broth.

Food Fermentation Practical (8) -Preparation of Idli

Ingredients: Idli preparation contains a number of different ingredients these are 1) Rice 2) Black gram dhal 3) Salt 4) water. Dehulled soybeans or Bengal gram can be used as a substitute for black gram dhal and a number of cereal grains can replace rice. However, there may be marked change in the texture and flavor when using substituted materials. It has been reported that rice variety and its physical characteristics are very important to produce a good quality idli. White Kar and IR20 varieties of rice have given much better performance in the production of idli, especially the White Kar variety because of its high amylose content, low amylopectin content better gelatinization, and better water uptake ability.

Proportion of cereal to legume: Ordinary idli consists of three parts rice and one part Black gram dhal plus salt to taste. Kancheepuram idli is prepared from one part rice and one part black gram dhal plus cashew nuts, ghee, salt, pepper, ginger and cumin added to taste. Normally a proportion of rice to black gram dhal varies from 4:1 to 1:4, the 2:1 being the best. It has been seen that when black gram dhal proportion is less than 25%, the steamed idli was hard and organoleptically unacceptable whereas when it is more than 50%, the product obtained is too sticky to be acceptable. Thus, not only can the ingredients be varied, but the proportions can also be varied within a wide range and still an acceptable product is obtained.

I. Procedure

White polished rice is carefully washed and soaked for 5-10 hours. Black gram dhal is carefully washed and soaked for 5-10 hours. The rice is then drained and coarsely ground in a stone mortar or other grinder. The black gram dhal is drained and finely ground in a stone mortar. The rice and black gram dhal slurries are combined to form a rather thick batter, which is stirred with hands. Salt is added to taste. Other seasonings, such as chilies are occasionally added. The batter is placed in a warm place to ferment overnight. In the morning, the batter is poured into the cups of an idli steamer, which is placed in a covered pan or cooker and steamed until the starch is gelatinized and the idli cakes are soft and spongy.

Food Fermentation Practical (9) -Preparation of Dosa

II. Ingredients

The ingredients used for dosa preparation are similar to as that of idli preparation i.e. rice, black gram dhal, salt and water. Rice may be substituted by wheat, bajra *Pennisetum typhoideum*, maize, or kodri and black gram dhal may be substituted by sprouted peas, cowpeas (Vigna catjang), field beans (Dolichos lablab) or soybeans. Fresh groundnut oilcakes may also be substituted for black gram dhal.

III. Soaking and batter formation

Generally, equal quantities of rice and dehulled black gram dhal are soaked in water at room temperature separately for 5-10 hours. It is common practice that finely ground powders are used to prepare batter. The finely ground powders are mixed with water at temperatures

ranging from 48° to 98 °C, best at 80 °C for the preparation of batter. Water is added in the range of 2.0 to 2.2 times the initial dry weight of ingredients to prepare a batter of viscosity desired for dosa. Salt is added from 0.8 to 1.0% as a seasoning before fermentation.

IV. Fermentation time

Traditionally, dosa batter is kept overnight for fermentation. The fermentation time should be sufficient to allow a definite leavening and acidification of the batter and to allow for the development of a pleasant acid flavor.

V. Incubation temperature

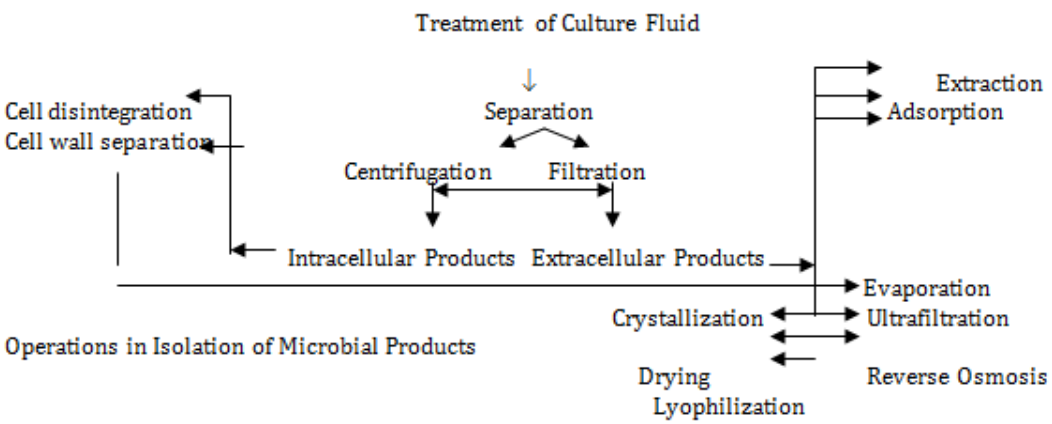
Ordinarily, the dosa fermentation is carried out at room temperature. In the tropics, this generally means a temperature of 25-30 °C.

VI. Harvesting and preservation

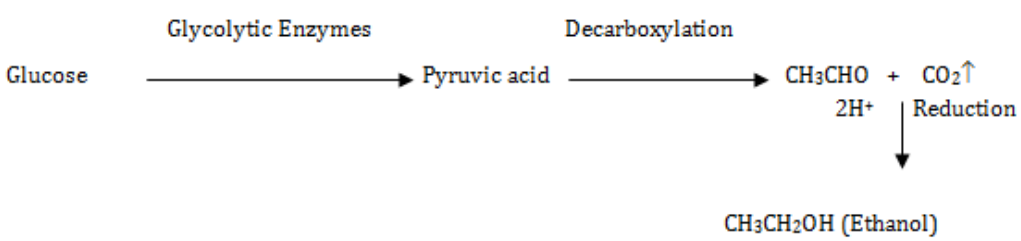
As soon as the batter becomes leavened and acidified, it is spread onto a hot and greasy griddle where it assumes the shape of a crisp pancake. The spreading of dosa on the griddle is an art that matures with practice. Sometimes a mixture of cooked different vegetable is poured onto the crisp dosa and the sides are rolled over it, which now becomes ready to be eaten with a spiced soup of dhal and vegetables popularly known as Samber. Food Fermentation Practical (10) -Production of Citric acid by *Aspergillus niger* in media containing molasses and sucrose.

VII. Theory

Many microorganisms like molds (*Aspergillus niger, A. awamori, Penicillium janthinallum, Trichoderma viridae, Mucor piriformis,* etc.), yeasts (*Candida lipolytica, C.tropicalis, C.citrica, Hansenula, Rodotorula, Pichia, Torulopsis* etc.) and bacteria (Bacillus licheniformis, Bacillus subtilis, Brevibacterium flavus, Corynebacterium species) have the capability to produce citric acid. Commercially spores of Aspergillus niger are employed to produce citric acid. It accumulates in culture solutions of pH 1.8-2.0. In fungi different metabolic pathways are involved in the production of citric acid but 78% of the citric acid is produced by the involvement of glycolysis and Tricarboxylic acid cycle. The acetyl COA derived by EMP pathway condenses with Oxaloacetate of Kreb's cycle to produce citric acid. Citric acid is an important chemical used in medicines, flavoring extracts, food and candies and in dyeing industry.



Flow Chart 16



VIII. Materials required

Molasses; Sucrose; Flasks; Test tubes; Sodium hydroxide; Ammonium dihydrogen orthophosphate; Trace element solution of composition: Zinc sulfate 3mg/100ml, Copper sulfate 680mg/100ml, Magnesium sulfate 2.0g/100ml and EDTA 2.0g/100ml; Dissolve in 100ml distilled water and incubate at 25oC for 3 days.

Composition of production medium of citric acid

Molasses or Sucrose 45gm/300ml, Ammonium dihydrogen orthophosphate 0.75gm/300ml, Potassium dihydrogen orthophosphate 0.3gm/300ml, Tween-80 0.6gm/300ml; pH for molasses 5.0-6.0 and for sucrose 2.0-3.0 Add trace element solution before sterilization. Sterilize at 15lb pressure for 15 minutes.

I. **Procedure**

Prepare two sets (one for molasses and second for sucrose) of 250ml flasks. Each set having three flasks containing 100ml production medium. One set having molasses and sucrose in the second set. Label the flasks of each set as control flask, Methanol containing flask, and flask without methanol. Add 3ml of methanol in the flask labeled as methanol flask. Sterilize both the sets at 15 lb pressure for 15 minutes. Cool the flasks and inoculate all the flasks of both the sets with spores of *Aspergillus niger* except the control flask. Incubate the flasks at 25 °C for 4-5 days on shaker. After the fermentation is over, filter the contents. Compare the acidity of the filtrate with the control flask.

II. **Acidity**

Titrate the filtrate against 0.1N NaOH using phenolphthalein as an indicator. Appearance of pink color is the end point. Note down the volume of filtrate used in titration and calculate the strength of citric acid in filtrate.

General calculations

NaOH Filtrate (citric acid)

$N_1 \times V_1 = N_2 \times V_2$

$0.1 \times 10 = N_2 \times B$ (say); $N_2 = 1/B$; B = Volume of filtrate used

Strength of citric acid = Equivalent weight of citric acid × Normality

$= 64.3 \times N_2 =$ 'Z' (say).