UNIT – 4
INDUSTRIAL ALCOHOL PRODUCTION

Alcohol fermentation was the first fermentation known to mankind. Distilleries began to appear in Europe in the middle of the seventeenth century. At first, alcohol was used only for human consumption. Later, the demand for alcohol as a universal solvent and chemical raw material developed. The distilling industry, therefore, grew very rapidly. The annual production in the United States alone has now reached one billion gallons.

Ethyl alcohol, CH_3CH_2OH (synonyms: ethanol, methyl carbinol, grain alcohol, molasses alcohol, grain neutral spirits, cologne spirit, wine spirit), is a colorless, neutral, mobile flammable liquid with a molecular weight of 46.47, a boiling point of 78.3 and a sharp burning taste.

Properties of Ethanol

Ethyl alcohol undergoes a wide range of reactions, which makes it useful as a raw material in the chemical industry. Some of the reactions are as follow:

(i) *Oxidation*: Ethanol may be oxidized to acetaldehyde by oxidation with copper or silver as a catalyst:

$$CH_3CH_2OH \xrightarrow{Cu,\ Ag} CH_3CHO + H_2$$

(ii) *Halogenation*: Halides of hydrogen, phosphorous and other compounds react with ethanol to replace the – OH group with a halogen:

$$3CH_3CH_2OH + PCl_3 \longrightarrow 3CH_3CH_2Cl + P(OH)_3$$

(iii) *Reaction with metals*: Ethanol reacts with sodium, potassium and calcium to give the alcoholates (alkoxides) of these metals:

$$2CH_3CH_2OH + 2Na \longrightarrow 2CH_3ONa + H_2$$

(iv) *Haloform Reaction*: Hypohalides will react with ethanol to yield first acetaldehyde and finally the haloform reaction:

$$CH_3CH_2OH + NaOCl \longrightarrow CH_3CHO + NaCl + H_2O$$
$$CH_3CHO + 2NaOCl \longrightarrow CCl_3CHO + 3NaOH$$
$$CCl_3CHO + NaOH \longrightarrow \underset{\text{Chloroform}}{CHCl_3} + HCOONa$$

(v) *Esters*: Ethanol reacts with organic and inorganic acids to give esters:

$$CH_3CH_2OH + HCl \longrightarrow \underset{\text{Ethylchoride}}{CH_3CH_2Cl} + H20$$

(vi) *Ethers*: Ethanol may be dehydrated to give ethers:

$$2CH_3CH_2OH \xrightarrow{Catalyst} CH_3CN_2OCH_2CH_3 + H_2O$$

(vii) *Alkylation*: Ethanol alkylates (adds alkyl-group to) a large number of compounds:

H_3SO_4: $CH_3CH_2HSO_4$ (ethyl hydrogen sulfate)
NH_3: $CH_3CH_2NH_2$ (ethyl amine)

Uses of Ethanol

(i) ***Use as a chemical feed stock*****:** In the chemical industry, ethanol is an intermediate in many chemical processes because of its great reactivity as shown above. It is thus a very important chemical feed stock.
(ii) ***Solvent use*****:** Ethanol is widely used in industry as a solvent for dyes, oils, waxes, explosives, cosmetics etc.
(iii) ***General utility*****:** Alcohol is used as a disinfectant in hospitals, for cleaning and lighting in the home, and in the laboratory second only to water as a solvent.
(iv) ***Fuel*****:** Ethanol is mixed with petrol or gasoline up to 10% and known as gasohol and used in automobiles.

Denatured Alcohol

All over the world and even in ancient times, governments have derived revenue from potable alcohol. For this reason when alcohol is used in large quantities it is denatured or rendered unpleasant to drink. The base of denatured alcohol is usually 95% alcohol with 5% water; for domestic burning or hospital use denatured alcohol is dispended as methylated spirit, which contains a 10% solution of methanol, pyridine and coloring material. For industrial purpose methanol is used as the denaturant. In the United States alcohol may be completely denatured (C.D.A. – completely denatured alcohol) when it cannot be used orally because of a foul taste or four smelling additives. It may be specially denatured (S.D.A. – specially denatured alcohol) when it can still be used for special purposes such as vinegar manufacture without being suitable for consumption.

Manufacture of Ethanol

Ethanol may be produced by either synthetic chemical method or by fermentation. Fermentation was until about 1930 the main means of alcohol production. In 1939, for example 75% of the ethanol produced in the US was by fermentation; in 1968 over 90% was made by synthesis from ethylene. Due to the increase in price of crude petroleum, the source of ethylene used for alcohol

production, attention has turned worldwide to the production of alcohol by fermentation.

The production of gasohol (gasoline – alcohol blend) appears to have received more attention than alcohol use as a feed stock. Nevertheless, the latter will also surely assume more importance if petroleum price continues to ride. Governments the world over have set up programs designed to conserve petroleum and to seek other energy sources. One of the most widely publicized programs designed to utilize a new source of energy is the Brazilian National Ethanol Program. Set-up in 1975, the first phase of this program aims at extending gasoline by blending it with ethanol to the extent of 20% by volume. The United States government also introduced the gasoline programme based on corn fermentation in 1980 following the embargo on grain sales to the then Soviet Union.

Substrates / Fermentation Medium / Raw material

A variety of carbohydrates available as waste-products of agricultural-industries are used for the preparation of fermentation medium. On the basis of their chemical nature, they may be grouped as:

(1) Saccharide materials—molasses, hydrol, whey, technical glucose, fruit juices

(2) Starchy materials—potato starch, corn starch, wheat flour, cereals (e.g. oats).

(3) Cellulosic material—sulphite waste liquor and others.

Apart from sucrose, other conventional fermentation substrates for ethanol fermentations include simple sugars derived from plants and dairy wastes. Use of root and tuber starch (cassava, potato, etc.) or grain starch (maize, wheat, rice, etc.) requires treatment before their use. For example, starchy material is subjected to saccharification, where starch gets hydrolysed, thus forming fermentable sugars, lactose and glucose.

The raw material used for the preparation of fermentation medium depends upon the agricultural produce of the country. For example, cane-molasses is used in India. Thus, in Brazil sugar cane, already widely grown in the country, is the major source of fermentation alcohol, while it is planned to use cassava and sweet sorghum. In the United States enormous quantities of

corn and other cereals are grown and these are the obvious substrates. Cassava grows in many tropical countries and since it is high yielding it is an important source in tropical countries where sugar cane is not grown.

Molasses is a source of sugar, and is used in many fermentation industries including the production of potable and industrial alcohol, acetone, citric acid, glycerol, and yeasts. It is a by-product of the sugar industry. There are two types of molasses depending on whether the sugar is produced from the tropical crop, sugar cane (*Saccharum officinarum*) or the temperate crop, beet (*Beta alba*).

Average composition of beet and cane molasses

	Beet Molasses % (W/W)	*Cane Molasses % (W/W)*
Water	16.5	20.0
Sugars:	53.0	64.0
Sucrose	51.0	32.0
Fructose	1.0	15.0
Glucose	-	14.0
Raffinose	1.0	-
Non-sugar (nitrogeneous Materials, acids, gums, etc.)	19.0	10.0
Ash	11.5	8.0

The optimum sugar concentration ranges between 10 to 18% in the fermentation medium. Also, a suitable source of nitrogen, such as ammonium sulphate, is added in optimum concentration. When beet-molasses is used, usually a small quantity of black-strap molasses is also added to meet the biotin deficiency in the beet-molasses. The pH of the medium is adjusted to 4.8 to 5. The pH can be adjusted by the use of H_2SO_4 or lactic acid. Alternatively, it can be adjusted by growing lactic acid bacteria prior to fermentation. It is not necessary to sterilize the fermentation medium. This is mainly due to highly acidic pH which hinders the growth of undesirable bacteria. However, pasteurization of the fermentation medium may be practiced.

Organism used and inoculums production

Alcohol-resistant yeasts, strains of *Saccharomyces cerevisiae* are used. In general the inocula are made of selected alcohol-tolerant yeast strains usually

Sacch. Cerevisiae grown aerobically with agitation and in a molasses base. Progressively larger volumes of culture may be developed before the desired volume is attained.

There are two types of *Saccharomyces* species, one is a top fermenter and the other a bottom fermenter. Top fermenter yeasts (e.g. *S. cerevisiae)* are active at the top surface of the fermenting liquid. They remain either in an evenly distributed, layer, or as a ring along the walls of the fermentation vat. Bottom fermenters (e.g. *S. carlsbergensis)* develop only at the bottom of the fermentation vats. They are whirled up in the liquid owing to carbon dioxide evolution

Species of the bacterial genus *Zymomonas*, such as *Z. mobilis*, which are Gram negative facultative anaerobes that normally ferment only glucose, fructose or sucrose. They afford greater ethanol yields than does *S. cerevisiae*, but are not as ethanol tolerant.

In future, alternate routes are likely to involve genetically engineered organisms that have the ability to utilize a wider range of carbon sources and have better fermentation properties. For example, *Escherichia coli*, which normally produces only relatively small amounts of ethanol, has been transformed with a plasmid that encodes the alcohol dehydrogenase and pyruvate decarboxylase from *Z. mobilis*. Such transformants produce ethanol under both aerobic and anaerobic conditions.

Preparation of Inoculum:

A large amount of yeast culture is required in the industrial production of ethyl alcohol. The size of inoculum ranges from 8-10 % with an average of 4 % volume by volume. The medium used in the preparation of inoculum is the same as that of the actual fermentation process. A high degree of aeration and agitation is necessary, since this favours rapid growth of yeast, producing a large amount of cell mass. The optimum pH is 4.8 to 5. The optimum temperature is 28° to 30°C. Steps involved in the building up of inoculum are:

(a) 10 ml of sterile medium in a test-tube is inoculated with pure yeast culture. The tube is incubated at 28° to 30°C for a suitable period.

(b) The tube culture is transferred to a flask, containing 200 ml of sterile medium. The flask is incubated at 28° to 30°C until pre-determined cell-mass is obtained.

(c) The flask culture is, in turn, transferred to a suitable glass container, containing about 4 litres of sterile medium and incubated for an appropriate length of time.
(d) Finally, the contents of the glass container are equally transferred into small tanks (yeast vessels) installed closely to a fermentor. Each small tank contains 10 to 40 gallons of sterile medium. After incubation the inoculum is ready for inoculating the fermentor. The inoculum is added to the fermentor by means of pumping or gravity. This operation of addition of inoculum to the fermentation medium is called 'pitching'.

Fermentation Conditions:

For the satisfactory progress and completion of the alcohol fermentation, the following conditions are carefully controlled:

Carbon Sources: Sugar concentration in the range of 10 to 18% is found satisfactory. Sugar concentration of 12% is often used. For example, cane molasses contains sucrose (cane sugar) as a carbon source. The sucrose content of this raw material is about 48 to 55%. Wort (molasses diluted with water) is prepared in-such a way as to contain about 10% sugars. High concentration affects yeast adversely while low concentration makes the process uneconomical.

Nitrogen Sources: Many organic, as well as inorganic nitrogenous compounds may be used in this fermentation. Ammonium sulphate (0-15 gms per 2-5 gallons of molasses) is generally used with success to supply nitrogen for growing yeast cells. The level of nitrogen in the medium is limited. Excess nitrogen is objectionable, since it promotes the growth of yeast, inhibiting the fermentation.

Growth Factors: It is not necessary to add the growth factors separately in the preparation of the fermentation medium, since the raw materials (e.g. cane-molasses) used satisfy the requirements of the culture.

pH: pH of the fermentation medium is adjusted to 4.8 to 5. The higher values of pH increase the chances of contamination, whereas lower pH values inhibit the yield of ethyl alcohol.

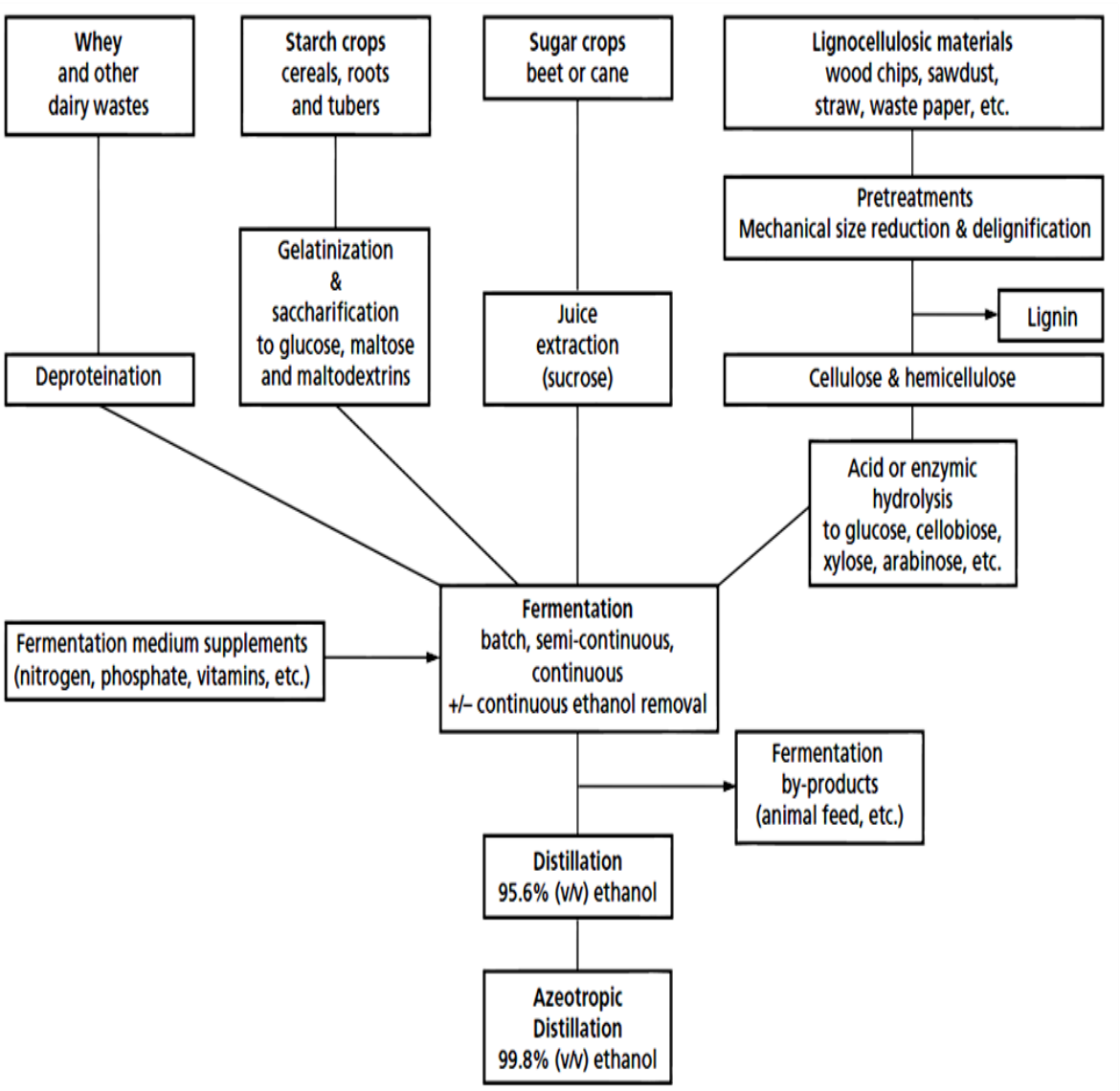
Temperature: Usually, the temperature range of 70° to 80°F (28 to 30^0 C) is preferred. As there is heat evolution during fermentation, the temperature in the fermentation tank rises gradually and is controlled by means of cooling coils. Temperature can also be controlled by cold water spray around the fermentation tank. Intermittent agitation is required for the uniform cooling of the medium. Higher temperatures favour the growth of bacteria and also cause loss of ethyl alcohol due to evaporation.

Time: Fermentation starts within a few hours after the addition of yeast. The process becomes very rapid after 24 hours to complete the fermentation. Duration for ethyl alcohol fermentation is usually 30 to 72 hours when the specific gravity of the fermented liquid (wash) becomes constant. At this stage it contains 6 to 8 % of ethyl alcohol.

Yield: On an average 0.4 gallons of ethyl alcohol is obtained from one gallon of molasses. Strict technical control is essential to ensure satisfactory yield. About 90% of carbohydrate is converted into alcohol.

Some of the techniques aimed at improving productivity are the following:

(i) Developments of new strains of yeast of *Saccharomyces uvarum* able to ferment sugar rapidly, to tolerate high alcohol concentrations, flocculate rapidly, and whose regulatory system permits it to produce alcohol during growth.
(ii) The use of continuous fermentation with recycle using the rapidly flocculating yeasts.
(iii) Continuous vacuum fermentation in which alcohol is continuously evaporated under low pressure from the fermentation broth.
(iv) The use of immobilized *Saccharomyces cerevisiae* in a packed column, instead of in a conventional stirred tank fermentor. Higher productivity consequent on a higher cell concentration was said to be the advantage.



Recovery:

The fermented liquid (wash, the fermented liquor or 'beer') is allowed to settle for a few hours, and then, distilled in analyser and rectifier columns to obtain rectified spirit (ethyl alcohol) and fusel oil (higher alcohols). The highest concentration that can be secured by fractional distillation is the constant boiling mixture. This mixture contains 95.6 % ethyl alcohol and remaining 4-4% water. The spent wash and bottom sludge are drained as distillery wastes. The product is marketed as rectified spirit, denatured spirit, or special denatured spirit. For the manufacture of rum, gin, whisky and brandy, matured rectified spirits used according to the ISI specifications. Different techniques of ageing, dilution and *blending* are employed for the manufacture of these potable drinks.

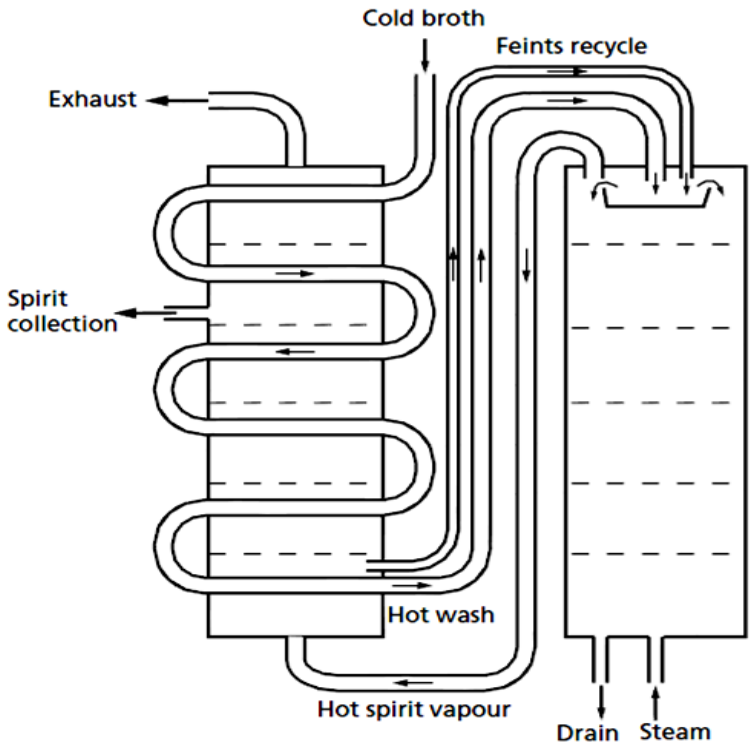
After fermentation the fermented liquor or 'beer' contains alcohol as well as low boiling point volatile compounds such as acetaldeydes, esters and the higher boiling, fusel oils. The alcohol is obtained by several operations.

- First, steam is passed through the beer which is said to be steam-stripped. The result is a dilute alcohol solution which still contains part of the undesirable volatile compounds.
- Secondly, the dilute alcohol solution is passed into the center of a multi-plate aldehyde column in which the following fractions are separated: esters and aldehydes, fusel oil, water, and an ethanol solution containing about 25% ethanol.
- Thirdly, the dilute alcohol solution is passed into a rectifying column where a constant boiling mixture, an azeotrope, distils off at 95.6% alcohol concentration.

Distillation

Distillation is used to recover fusel alcohol, acetone and other solvents from fermentation media, and for the preparation of potable spirits. Batch distillation in pot stills continues to be used for the production of some whiskies, but for most other purposes continuous distillation is the method of choice. With ethanol, for example, the continuous system produces a product with a maximum ethanol concentration of 96.5% (v/v).

Some continuous stills may be in the form of four or five separate columns, but the Coffey-type still comprises just two columns, the 'rectifier' and 'analyser', each containing a stack of 30–32 perforated plates (Fig.). Incoming fermentation broth is heated, as it passes down a coiled pipe within the rectifier column, by the ascending hot vapour produced by the analyser column. The now hot broth is released into a trough at the top of the analyser column and as it falls down the column it is heated by steam. Hot vapours generated are then conveyed from the top of the analyser column to the bottom of the rectifier column. As it passes upwards it is condensed on the coils carrying incoming broth. There is a temperature gradient in the rectifier column and each volatile compound condenses at its appropriate level, from where the fraction is collected.

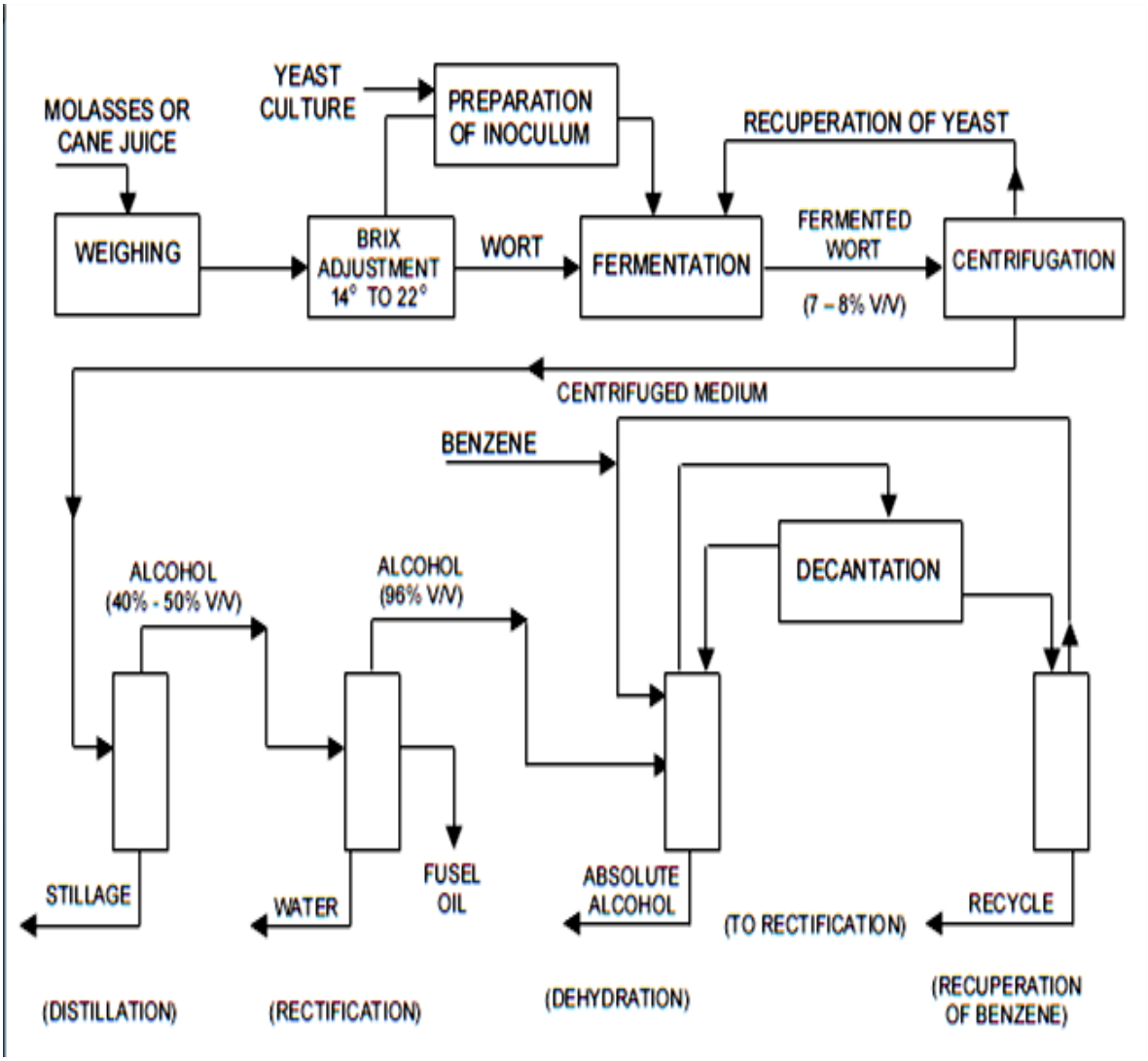


A Coffey-type still (only six of the 30 column sections shown)

Azeotropic distillation: To obtain 200° proof alcohol, such as is used in gasohol blending, the 96.58% alcohol is obtained by azeotropic distillation. The principle of this method is to add an organic solvent which will form a ternary (three-membered) azeotrope with most of the water, but with only a small proportion of the alcohol.

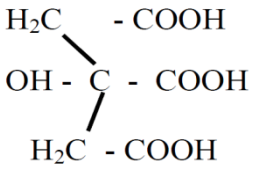
Benzene, carbon tetrachloride, chloroform, and cyclohexane may be used, but in practice, benzene is used. Azeotropes usually have lower boiling point than their individual components and that of benzene-ethanol-water is 64.6°C. On condensation, it separates into two layers. The upper layer, which has about 84% of the condensate, has the following percentage composition: benzene 85%, ethanol 18%, water 1%. The heavier, lower portion, constituting 16% of the condensate, has the following composition: benzene 11%, ethanol 53%, and water 36%.

In practice, the condensate is not allowed to separate out, but the arrangement of plates within the columns enables separation of the alcohol. Four columns are usually used. The first and second columns remove aldehydes and fuel oils, respectively, while the last two towers are for the concentration of the alcohol.


MOLASSES OR
CANE JUICE
YEAST
CULTURE
PREPARATION
OF INOCULUM
RECUPERATION OF YEAST
WEIGHING
BRIX
ADJUSTMENT
14° TO 22°
WORT
FERMENTATION
FERMENTED
WORT
(7 – 8% V/V)
CENTRIFUGATION
CENTRIFUGED MEDIUM
BENZENE
DECANTATION
ALCOHOL
(40% - 50% V/V)
ALCOHOL
(96% V/V)
STILLAGE
WATER
FUSEL
OIL
ABSOLUTE
ALCOHOL
(TO RECTIFICATION)
RECYCLE
(DISTILLATION)
(RECTIFICATION)
(DEHYDRATION)
(RECUPERATION
OF BENZENE)

CITRIC ACID PRODUCTION

Citric acid has structure like



Citric acid is an intermediate in the citric acid cycle (TCA)

Uses of Citric Acid

Citric acid is used in the food industry, in medicine, pharmacy and in various other industries.

Uses in the food industry

i) Citric acid is the major food acidulant used in the manufacture of jellies, jams, sweets, and soft drinks.
ii) It is used for artificial flavoring in various foods including soft drinks.
iii) Sodium citrate is employed in processed cheese manufacture.

Uses in medicine and pharmacy

i) Sodium citrate is used in blood transfusion and bacteriology for the prevention of blood clotting.
ii) The acid is used in efferverscent powers which depend for their efferverscence on the CO_2 produced from the reaction between citric acid and sodium bicarbonate.
iii) Since it is almost universally present in living things, it is rapidly and completely metabolized in the human body and can therefore serve as a source of energy.

Uses in the cosmetic industry

i) It is used in astringent lotions such as aftershave lotions because of its low pH.
ii) Citric acid is used in hair rinses and hair and wig setting fluids.

Miscellaneous uses in industry

i) In neutral or low pH conditions the acid has a strong tendency to form complexes hence it is widely used in electroplating, leather tanning, and in the removal of iron clogging the pores of the sand face in old oil wells.

ii) Citric acid has recently formed the basis of manufacture of detergents in place of phosphates, because the presence of the latter in effluents gives rise to eutrophication (an increase in nutrients which encourages aquatic flora development).

Before the development of fermentation technology, citric acid was obtained by extraction from the juices of certain citrus fruits (e.g. lemon), and later from pineapple wastes. Today most of the commercial citric acid is obtained by a microbial fermentation process. There are various fermentation processes used in the manufacture of citric acid.

(a) **Surface-culture process**
(b) **Submerged-culture process using** *Aspergillus niger*
(c) **Submerged-culture process using a yeast**

(a) **Surface-culture process for citric acid production**

In this process, culture is inoculated across the surface of the production medium. In addition, culture remains on the surface throughout the fermentation.

Organism:

Many fungi have been reported to possess the ability to produce citric acid but *Aspergillus niger* strains among these fungal strains are used for the following reasons:

(i) They are efficient (i.e. high-yielding) strains, (ii) They possess fairly uniform biochemical properties, (iii) They produce a negligible or small amount of oxalic acid (iv) They can easily be cultivated.

Preparation of inoculum*:*

The spores of *Aspergillus niger* strain required to inoculate are produced by growing the fungus from a stock culture on a suitable solid sporulation medium at 25°C for 4 to 14 days. The trace amounts of manganese salts, zinc or iron salts are added. Suspension of spores is obtained by suspending the grown spores in a suitable diluent, such as water containing a wetting agent. The spores of inoculum are added to the production medium so as to keep them floating on the surface, since this is a surface-culture process. This may be accomplished by a suitable special inoculating device.

Source of carbon:

Sucrose is the best source of carbon among various tested organic substances, particularly sugars, in producing high yields of citric acid. It was also reported that sucrose concentration exceeding 15 per cent should not be used, since the excess amount of sugar (less than 3 per cent) remained unconverted to citric acid. When a part of sucrose was substituted by fructose or glucose, it resulted in lower yields of citric acid than when controls containing sucrose alone were used. Therefore, precaution is to be taken while using sucrose to avoid partial hydrolysis.

Beet molasses or cane molasses is extensively used as a carbon substrate in the fungal production of citric acid on a commercial basis. Beet molasses requires pretreatment, since it contains excessive amounts of trace metals. Therefore, ferrocyanide or ferricyanide may be added to the production medium before sterilization. The metals of the beet molasses form a complex with the added chemical agent, thus eliminating themselves as the precipitate. Alternatively, the clarified molasses may be passed through a cation-exchange resin. Under laboratory conditions, the highest yields of citric acid are secured, provided sucrose has been passed through an ion-exchange resin.

Inorganic salts:

Apart from the carbon, hydrogen and oxygen supplied by the added carbohydrate, the trace metals, namely nitrogen, potassium, phosphorus, sulphur, and magnesium are needed in the fermentation medium used for citric acid production.

Fermentation medium by Currie

Component	***Gms /Litre***
Sucrose	125 - 150
NH_4N0_3	2.0 – 2.5
KH_2PO_4	0.75 – 1.0
$MgS0_4 . 7H_2O$	0.20 – 0.25
pH with HCl	3.4 to 3.5

Fermentation medium by Doelger and Prescott

Component	***Gms /Litre***
Sucrose	140
NH_4N0_3	2.2
KH_2PO_4	1.0
$MgS0_4 . 7H_2O$	0.23
pH with HCl	1.5 to 2.2

Sterilization: 10 lbs steam pressure per square inch for 30 minutes

Metallic salts beyond their limits affect the fermentation adversely and their concentration is different for different strains. Calcium carbonate is not added to the production medium to bring about the neutralization of citric acid produced because its presence favours contamination. Moreover, its absence favours higher yields of citric acid and a shorter fermentation time.

Pans or trays:

These should be made up of aluminium or stainless steel because due to acidic pH of the medium corrosion of other metals takes place and also iron from iron trays affect fermentation.

Production medium is placed in shallow pans in such a way that a shallow layer of medium with a depth of 1 to 2-5 cm. (but sometimes up to 8 cm.) is formed.

Temperature:

The exact required temperature of incubation depends in part on the fungal strain and the fermentation conditions/According to Doelger and Prescott, a temperature range of 26 to 28°C is considered to be satisfactory.

Ratio of surface area to volume:

This is an important bioparameter in this process, since the rate of bioconversion of sugar to citric acid depends on the ratio of surface area to the volume of the medium. The lower the ratio of the volume to surface area, the higher is the yield of citric acid. In this shallow-pan method, a large surface area of the mycelial mat is exposed to a relatively shallow layer of a production medium. Under such conditions, high-level bioconversions of sugar occur. In this respect, this process is superior to the submerged-culture process.

Aeration:

It is necessary to supply air to the surface of the seeded medium. From laboratory studies it has been found that, the rate of aeration, either higher or lower than the optimum rate gives lower yields of citric acid. Therefore, it is essential to determine the rate of the air supply required for each new apparatus installed.

Time:

For the shallow-pan method (i.e. surface-culture process) the period required for the progress and termination of the fermentation process is in the range of 7 to 10 days.

Yields: It has been reported that yields of citric acid may be in the range of 60 to 80 gms (or higher) of anhydrous citric acid per 100 gms of incorporated sugar.

Wells, Moyer and May obtained a maximum yield of 90.7 per cent of citric acid from glucose on the basis of the sugar consumed.

(b) Submerged-culture process for citric acid production by using *Aspergillus niger*

Organism used: *Aspergillus niger*

Medium: molasses medium, medium that consists of pure sucrose or a solution of cation-depleted molasses, glucose solutions derived directly from corn have been employed.

Inoculum production:

The mold spores are produced under controlled aseptic conditions. When harvested, they are used in specific quantities to seed the inoculum tank containing a medium designed to develop cellular mass and to control morphology rather than to produce citric acid. Then it is transferred to the production tank under aseptic conditions.

Fermentation conditions

In this process the fungus is grown dispersed through a liquid production medium. Usually, fermentation is carried out in the fermentation vessel consisting of a sterilizable tank of the capacity of thousands of gallons. The fermentation tank is equipped with a mechanical agitator and a sparger.

In contrast to the composition of the inoculum medium, production medium contains constituents so as to favour citric acid production rather than growth. High aeration rates (i.e. 0.5 to 1.5 volumes per minute) producing much foams are necessary.

Samples are regularly withdrawn to determine the citric acid and sugar content revealing the progress of the fermentation process. In addition to this, pH, dissolved oxygen and solids content are determined. It has been reported that yields of citric acid from such sugar substrates may exceed 90 per cent.

(c) Submerged-culture process for citric acid production by using yeast

Organism used: species of yeast e.g. *Candida guilliermondii, Candida lipolytica*, *Candida lipolytica* ATCC 20114

Medium:

Medium containing either glucose or black-strap molasses with an equivalent amount of sugar, hydrocarbons (C_9 to C_{20}) normal paraffins for *Candida lipolytica*, chemical agent monofluoroacetate in media for *Candida lipolytica* ATCC 20114.

Fermentation conditions:

Grown submerged in a medium containing either glucose or black-strap molasses with an equivalent amount of sugar. With this fermentation, the duration of the fermentation period was shorter than that of the submerged-culture process involving *A. niger*. A subsequent improvement patent quotes citric acid concentrations of 110 gms./ltr.

Using certain strains of *Candida lipolytica,* the possibility of using hydrocarbons as a raw material in citric acid fermentation has been shown. A patent was issued in 1970 for the byconversion of C_9 to C_{20} normal paraffins to citric acid by *C. lipolytica.* Thus, citric acid weight yields exceeding 100 per cent were claimed. In 1972 other patents were issued describing a method to select high-yielding mutant strains of *C. lipolytica*. By using a chemical agent, monofluoroacetate, in media, Japanese workers have succeeded in isolating mutants with only one hundredth of the cis-aconitase activity exhibited by the parent strain *(C. lipolytica* ATCC 20114). These strains are able to accumulate 112 gms of citric acid per litre after 3 days' incubation at 30°C. On the basis of the substrate consumed, the yield is 145 per cent. In 1974 Pfizer patented a continuous process for *C. lipolytica*-citric acid fermentation. The process utilizes a single vessel. Continuous feeding of paraffin and continuous withdrawal of fermented broth are to be practised.

Recovery of citric acid:

The recovery operation for citric acid from the harvested fermentation broth is difficult due to the presence of unconverted sugars, the presence of

other acid fermentation products (e.g. oxalic acid) and the presence of trace salts as impurities.

Procedure of recovery

i) The fermentation liquor is drained off to separate the mycelium. In addition, any intracellular citric acid present in the mycelium is obtained by pressing the mycelial mat.
ii) The recovered fermentation liquor is treated with milk of lime forming the precipitate of calcium citrate.
iii) The precipitate of calcium citrate is filtered and washed.
iv) Calcium citrate is treated with an equivalent of sulphuric acid to liberate citric acid, leaving behind the precipitate of calcium sulphate.
v) The precipitated solution is filtered and washed.
vi) An impure solution of citric acid is subjected to decolorization by the activated carbon. Then, it is also demineralized.
vii) Finally, the pure citric acid solution is evaporated and is crystallized from the solution.

The alternative method Counter current extraction method.

i) Organic solvents (100 parts of tri-n-butyl phosphate and 5 to 30 parts of n-butyl acetate are employed for the purpose of extracting citric acid.
ii) Then citric acid is extracted from the organic phase with lime water.
iii) Lastly, the precipitate of calcium citrate is treated with sulphuric acid to liberate citric acid, leaving behind the precipitate of calcium sulphate.

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PRODUCTION OF ENZYMES

Enzymes are organic compounds which catalyze all the chemical reactions of living things – plants, animals and microorganisms. They contain mainly protein; some of them however contain non-protein components, prosthetic groups. When excreted or extracted from the producing organism they are capable of acting independently of their source. It is this property of independent action which drew early attention to their industrial use. Most industrial enzymes are obtainable from microorganisms.

USES OF ENZYMES IN INDUSTRY

Most of the enzymes used in industry are hydrolases (i.e., those which hydrolyze large molecules). In particular amylases, proteases, pectinases, and to a lesser extents lipases have been most commonly used.

(i) ***Production of nutritive sweeteners from starch*****:** The sweeteners which have been produced from starch are high conversion (or high DE) syrup, high maltose syrup, glucose syrup, dextrose crystals and high fructose syrup.

(ii) ***Proteolytic enzymes in the detergent industry*****:** The detergent industry is at present one of the greatest consumers of enzymes, and uses mostly proteases. Blood and pus stains from hospital linen and other protein dirt precipitate and coagulate on clothes and are ordinarily difficult to remove. The inclusion of proteolytic enzymes in a detergent or washing soap greatly facilitates the removal of such stains.

(iii) ***Microbial rennets*****:** Rennin is an acid protease found in gastric juice of young mammals where it helps to digest milk. It is used in the manufacture of cheese.

(iv) ***Lactase*****:** Lactase hydrolyzes the disaccharide lactose into its component galactose and glucose, both of which are sweeter than lactose and correspond to the addition of 0.9% sucrose. Thus, dairy products containing lactose, such as yoghurt, and ice cream, can be sweeter.

(v) ***The textile industry*****:** In the textile industry large amounts of starch, gelatin and their derivatives such as glue are used to strengthen threads (yarns) of synthetic and natural materials (e.g. cotton). At the end of the manufacturing the starch is removed with thermostable amylases. Natural silk threads Sericin is removed with a neutral proteolytic enzyme;

(vi) ***Pectinases for use in fruit juice and wine manufacture:***

(vii) ***Enzymes in the baking industry*****:**

(viii) ***Enzymes in the alcoholic beverages industry*****:**

(ix) ***Leather baiting*****:**

(x) ***Some medical uses of microbial enzymes*****: Papain and diastase**

Production of α Amylase (Fungal)

Various fungi and bacteria produce α amylases which are not identical. These are used in preparation of sizing agents and removal of starch sizing from woven cloth, preparation of starch sizing pastes for use in paper coating, manufacture of corn and chocolate syrups, production of bread, removal of food spots in dry cleaning and brewing industry. Bacterial amylase can be active at 55^0 C.

Surface culture method

Medium: Wheat or Rice bran

Organism:

Fungus *Aspergillus oryzae* and bacterium *Bacillus subtilis*

Procedure

This system, also known as the 'Koji' or 'moldy bran' method of 'solid state' fermentation is still widely used in Japan. The medium consists of moist sterile wheat or rice bran acidified with HCl; mineral salts including trace minerals are added. An inducer is also usually added; 10% starch is used for amylase, the desired pH is adjusted with acid, medium is sterilized and spread in relatively thin layers in metal trays up to a depth of 1 – 10 cms under aseptic conditions or placed in a revolving drum. Spores of *A. oryzae* are inoculated on it. A series of trays are enclosed in a large vessel. Aeration is done by circulating humidified air over the surface. Moisture (about 8%) is maintained by occasionally spraying water on the trays and by circulating moist air over the preparation. The temperature of the bran is kept at about 30°C by the circulating cool air. The production period is usually 30-40 hours, but could be as long as

seven days. The optimum production is determined by withdrawing the growth from time to time and assaying for enzyme.

Submerged culture method

Nowadays submerged culture methods are widely used.

Organism: Fungus *Aspergillus niger*

Medium: Starch salt medium

Component	Amount (g / litre)
Corn starch	24
Corn-steep liquor	36
KCl	0.2
Na_2HPO_4	47
$CaCl_2$	1
$MgCl_2.6H_20$	0.2

Procedure

Fermentor is a cylindrical tank of stainless steel of capacity 1000 to 30,000 gallons equipped with an agitator, aerating device, cooling system, foam control, pH & temperature control.

The production medium should contain energy, carbon and nitrogen source and special growth requirements such as amino acids and vitamins. Certain compounds induce or inhibit enzyme production. E.g. lactose induces and glucose inhibits amylase production, hence lactose should be added and glucose level should be maintained low. pH is kept neutral by adding buffer system of phosphates or calcium carbonate.

Sterilization of medium is done in fermentor at 121^0C for 15 to 20 minutes. After sterilization cooling is done and spores are inoculated. The oxygen requirement is usually high as most of the organisms employed in enzyme production are aerobic. Vigorous aeration and agitation are therefore done in the submerged fermentations for enzyme production. Batch fermentation is usually employed in commercial enzyme fermentation and lasts from one to seven days. *A. niger* produces α amylases in the 72 hours.

Enzyme Extraction

In order to limit contamination and degradation of the enzyme the broth is cooled to about 20°C as soon as the fermentation is over. Stabilizers such as calcium salts, proteins, sugar, and starch hydrolysates may be added and destabilizing metals may be removed with EDTA. Antimicrobials if used at all are those that are normally allowed in food such as benzoates and sorbates. Most industrial enzymes are extra-cellular in nature. In the case of cell bound enzymes, the cells are disrupted before centrifugation and /or vacuum filtration.

The extent of the purification after the clarification depends on the purpose for which the enzyme is to be used. Sometimes enzymes may be precipitated using a variety of chemicals such as methanol, acetone, ethyl alcohol or ammonium sulfate. The precipitate may be further purified by dialysis, chromatography etc., before being dried in a drum drier or a low temperature vacuum drier depending on the stability of the enzymes to high temperature. Ultra-filtration separation technique based on molecular size may be used.

Baker’s yeast / Brewer's yeast.

Baker's yeast is the common name for the strains of yeast commonly used as a leavening agent in baking bread and bakery products, where it converts the fermentable sugars present in the dough into carbon dioxide and ethanol. Baker's yeast is also called *Saccharomyces cerevisiae*, which is the same species commonly used in alcoholic fermentation, and so is also called **brewer's yeast**.

History

It is known when yeast was first used to bake bread, thought to be in Ancient Egypt. The first records that show this use came from Ancient Egypt. Researchers speculate that a mixture of wheat flour meal and water was left longer than usual on a warm day and the yeasts that occur in natural contaminants of the flour caused it to ferment before baking. The resulting bread would have been lighter and tastier than the previous hard flatbreads.

Refinements (improvements) in microbiology following the work of Louis Pasteur led to more advanced methods of culturing pure strains. In 1879 Great Britain introduced specialized growing vats for the production of *S. cerevisiae*, and in the United States around the turn of the century centrifuges were used for concentrating the yeast, making modern commercial yeast possible, and turning yeast production into a major industrial endeavor (attempt). The slurry yeast made by small bakers and grocery shops became cream yeast, a suspension of live yeast cells in growth medium, and then compressed yeast, the fresh cake yeast that became the standard leaven for bread bakers in much of the Westernized world during the early 20th century.

During World War II Fleischmann developed a granulated active dry yeast for the United States armed forces, which did not require refrigeration and had a longer shelf life and better temperature tolerance than fresh yeast; it is still the standard yeast for US military recipes. The company created yeast that would rise twice as fast, cutting down on baking time.

Types of baker's yeast

Active dried yeast, a granulated form in which yeast is commercially sold.

Baker's yeast is available in a number of different forms, the main differences being the moisture contents. Though each version has certain advantages over the others, the choice of which form to use is largely a question of the requirements of the recipe at hand and the training of the cook preparing it. Dry yeast forms are good choices for longer-term storage, often lasting several months at room temperatures without significant loss of viability. With

occasional allowances for liquid content and temperature, the different forms of commercial yeast are generally considered interchangeable.

- **Cream yeast** is the closest form to the yeast slurries of the 19th century, being essentially a suspension of yeast cells in liquid.
- **Compressed yeast** is essentially cream yeast with most of the liquid removed. It is soft solid, beige (light brown) in color, and arguably (possibly) best known in the consumer form as small, foil-wrapped cubes of **cake yeast**.

- **Active dry yeast** is the form of yeast most commonly available to noncommercial bakers in the United States. It consists of coarse oblong granules of yeast, with live yeast cells encapsulated in a thick jacket of dry, dead cells with some growth medium.
- **Instant yeast** appears similar to active dry yeast, but has smaller granules with substantially higher percentages of live cells per comparable unit volumes.
- **Rapid-rise yeast** is a variety of dried yeast (usually a form of instant yeast) that is of a smaller granular size, thus it dissolves faster in dough, and it provides greater carbon dioxide output to allow faster rising.

For most commercial uses, yeast of any form is packaged in bulk (blocks or freezer bags for fresh yeast; vacuum-packed brick bags for dry or instant); however, yeast for home use is often packaged in pre-measured doses, either small squares for compressed yeast or sealed packets for dry or instant.

Production of Baker's Yeast

Yeast Strain Used

Nowadays specially selected strains of *Saccharomyces cerevisiae* are used. Yeast strains used for the modern fast-rising dough have been developed with the following traditional and new physiological properties in mind.

(a) ability to grow rapidly at room temperature of about 20-25°C;
(b) easy dispensability in water;
(c) ability to produce large amounts of CO_2 in flour dough, rather than alcohol;
(d) good keeping quality, i.e., ability to resist autolysis when stored at 20°C;
(e) high potential glycolytic activity;
(f) ability to adapt rapidly to changing substrates;

(g) high invertase and other enzyme activity to hydrolyze the higher glucofructans rapidly;
(h) ability to grow and synthesize enzymes and coenzymes under the anaerobic conditions of the dough;
(i) ability to resist the osmotic effect of salts and sugars in the dough;
(j) high competitiveness i.e. high yielding in terms of dry weight per unit of substrate used.

Substrate / Fermentation medium

The substrate usually used for baker' yeast production is molasses. Where these are not available or are too expensive any suitable sugar-containing substrate e.g. corn steep liquor may of course be used. In the Soviet Union for example sulphite liquor is used for both alcohol and baker's yeast production. Beet and cane molasses, when they are simultaneously available, are treated separately: clarified, pH adjusted and sterilized. They are then mixed in equal amounts so that the nutritional deficiency of one type is made up by the other.

Cane sugar is particularly richer in biotin, panthothenic acid, thiamin and magnesium and calcium; while beet molasses is much richer in nitrogen. The molasses is clarified to remove inert colored material arising from colloidal particles and which can impart undesirable color to the yeast. Clarification may be achieved by precipitation with alum or calcium phosphate or by poly-electrolyte flocculating agents such as alginates and polyacrylamides. Clarification also helps to reduce foaming. 'Sterilization' is achieved by heating at 100°–110°C for about an hour, after the pH has been adjusted to pH 6-8 to prevent caramelization of the sugar.

Phosphorous, ammonium and smaller amounts of magnesium, potassium, zinc, and thiamin are added for maximum productivity to the mixed molasses. Antifoam is sometimes added.

1lb dry wt. of yeast would require
(lb = pound 1 kg = 2.2 kg 1lb = 0.453 kg)
4.3 lb of molasses (1.9 kg)
1.1 lb of $(NH_4)_2 SO_4$ (0.498 kg)
0.9 lb of ammonia (0.40 kg)
0.3 lb of $NH_4H_2PO_4$ (0.136 kg)
60 lb of air. (27.2 kg)

Fermentor processes

The fermentor for baker's yeast propagation is nowadays made of stainless steel. The fermentor may be anything from 75 to 225 cubic meters. Of this about 75% is occupied by the medium, the unused space being allowed for foaming. Generally, baker's yeast fermentors are aerated only by spargers which are so arranged that large volumes of air pass through per unit time: about one volume of air per volume of broth per minute. Spargers of different types are available. It is most important that aeration be high and constant. When the oxygen falls below 0.2 ppm anaerobic conditions set in and alcohol is formed.

The aeration through sparger holes is started as soon as mixing begins in the steam sterilized fermentor. Water, mineral nutrients, yeasts and the blended molasses containing 1% glucose are mixed. The amount of blended molasses added is calculated so that the total sugar in the fermentor does not exceed 0.1%. The pH is maintained at pH 4-6 by the addition of alkali and the temperature at 30°C by cooling. The amount of molasses to be added at predetermined intervals is arrived at by experimentation. Automatic sensoring and self-adjusting equipment for temperature pH, aeration, sugar, etc., are built into some modern fermentors. Large amounts of heat are evolved and the cooling of the fermentor is very important.

The period of fermentation in the trade or production fermentor varies from 10 to 20 hours depending on how much yeast is pitched into it; cells form from 3.5% to 5% dry wt. of the broth.

Harvesting the yeast

The fermentation broth is cooled and cells concentrated in centrifugal separators; they are washed by resuspension in water and centrifugation until they are lighter in color. The yeast cream resulting from this treatment contains 15-20% yeast cells. It is further concentrated by passing over a rotary vacuum filter or through a filter press. Sometimes the Mautner process is used to ensure a friable dry cream during vacuum filtration. This latter process consists of adding before filtration 0.2-0.6% (w/v) sodium chloride, which causes cell shrinking by osmosis. Excess salt is removed during filtration by spraying water

over the filtered yeast, so that the cells swell again. The resulting product has a dry matter content of 28-30%.

Packaging

Baker's yeasts may be packaged as moist (compressed) yeasts or as dried active yeast.

(i) *Compressed yeast*: The yeast product obtained after harvesting, is mixed with fine particles of ice, starch, fungal inhibitors and processed vegetable oils (e.g. glyceryl monostearate) which all help to stabilize it. It is then compressed into blocks of small (1-5 lb) blocks for household use or large (up to 50 lb) for factory bakery operations, stored at – 7 to 0°C and transported in refrigerated vans.

(ii) *Active dry yeast*: Dry yeast is more stable in that it can be used in areas or countries where refrigeration is not available. In many developing countries baker's yeast is imported from abroad in the form of active dry yeast. For active dry yeast production special strains better suited for use and dry conditions may be used. It has been found that when regular strains are used they perform better as dry yeasts when they are subjected to a number of treatments. These treatments include raising the temperature to 36°C (from about 30°C) towards the end of the fermentation, addition of alcohol-containing spent broth (resulting from centrifugation or finished yeast fermentation), synchronization of budding by alternate feeding and starving. The reason for the benefit is not known.

Yeast cream of 30-38% content from filter pressing is extruded through a screen to form continuous thread-like forms. These are then chopped fine and dried, using a variety of driers: tray driers, rotary drum driers, or fluidized bed driers. The final product has a moisture content of about 8% and may be packaged in nitrogen-filled tins. Sometimes anti-oxidants may be added to the yeast emulsion to further ensure stability.

Biofertilizers – (Azo, Rhizo, and PSB)

1) General account of microbes used as bio-fertilizers

Bio-fertilizers are the fertilizers containing microorganisms which when added in the soil; mineralize essential elements like Nitrogen, Sulphur, and Phosphorous, which are used by the plants and thus increase the crop yield.

Most of the mineral supply of soil is dependent upon microorganisms. The plants are unable to use the gaseous nitrogen present in the atmosphere or sulphur or phosphorous present in the soil. Such essential elements are to be converted into usable forms by plants **(Mineralization).** This conversion of organic compounds into simple inorganic compounds or into their constituent elements is called **mineralization.**

The bio-fertilizers do not directly increase soil fertility but they usually initiate or accelerate the process of mineralization.

The bio-fertilizers include—

I) Nitrogen fixing microorganisms
II) Phosphate solubilizing microorganisms

III) Sulphur oxidizing microorganisms

IV) Organic matter decomposing microorganisms

Bio-fertilizers are also known by the names—

- ➔ Microbial preparations
- ➔ Microbial cultures
- ➔ Microbial inoculants

I) Nitrogen fixing microorganisms

A large number of microorganisms are known to have the ability to reduce atmospheric nitrogen into nitrogenous compounds. **E.g. Ammonia**.

Nitrogen fixation: - The reduction of atmospheric gaseous nitrogen to ammonia is called as nitrogen fixation.

This nitrogen fixation can be studied under three headings—

A) Non-symbiotic N_2 fixation

B) Associative N_2 fixation

C) Symbiotic N_2 fixation

A) Non-symbiotic N_2 fixation: -

In this type, N_2 fixation is done by those microbes, which live freely and independently in the soil.

E.g. **Bacteria: -** *Azotobacter, Bacillus, Clostridium, Chlorobium, Chromatium, Desulfovibrio, Enterobacter, Escherichia coli, Klebsiella, Rhodospirillum, Thiobacillus spp etc.*

Cyanobacteria (Blue-green algae): - *Anabaena, Nostoc*

B) Associative N_2 fixation: -

In this type of association no nodules are formed like symbiotic bacteria. The bacteria grow in the **rhizosphere** in close contact with the roots; some times invade the outer cortical regions of the roots and fix nitrogen.

Azospirillum brazilense bacterium discovered by Brazilian microbiologist: J. Dobereniner (1978) is the best-studied bacterium forming associative symbiosis with the cereal roots. Others are—

Bacillus, Enterobacter, Klebsiella, Pseudomonas azotogensis.

These microbes fix nitrogen in association with the roots of grasses and cereal plants.

C) Symbiotic N_2 fixation

There are 3 types—

1. **Through nodule formation in legumes**
2. **Through nodule formation in non-legumes** (Actinomycetes symbiosis)
3. **Through non-nodulation** (Mycorrhizal symbiosis)

1. Through nodule formation in legumes

Symbiotic nitrogen fixation is accomplished by ***Rhizobium spp***, which occur upon the roots of ***leguminous plants.*** Before these bacteria can fix

nitrogen, they must establish themselves in the root cortical cells of the host plant ultimately forming **'root nodules'.**

2. Through nodule formation in non-legumes

(Actinomycetes symbiosis)

Nitrogen fixing symbiosis between ***non-leguminous trees*** and ***actinomycetes*** occur in almost **140** species woody perennial shrubs and trees belonging to **17** genera in **8** families. **'Alnus'** species in tem regions and **casuarina** species in the tropics and subtropics are capable of fixing atmospheric nitrogen by virtue of symbiotic relationship established between roots of tree and the soil microorganism **frankia.**

3. Through non-nodulation (Mycorrhizal symbiosis)

Mycorrhizal association is the most widely spread symbiosis among plants and most nitrogen fixing trees form abundant mycorrhizae on their roots.

Thus mycorrhiza is mutualistic symbiosis between roots of higher plants and fungal hyphae. In this association plants are benefited by uptake of **phosphorous and other inorganic nutrients,** enhance growth of tree.

II) Phosphate solubilizing microorganisms

After the decomposition of organic phosphorous containing compounds, inorganic compounds like phosphoric acid which combines with soil bases and produce salts of **calcium, magnesium, iron** are produced. These salts are less soluble and less available to plants.

These salts are converted into soluble form by the acids like **sulphuric and nitric acid** by the microorganisms. The **Tri-calcium phosphate** is transformed first into mono-phosphates and then into phosphates, which are absorbed by roots of plants.

These phosphate-solubilizing microbes are used as bio-fertilizers e.g.

Aspergillus, Flavobacterium, Penicillium, Pseudomonas, Micrococcus, Mycobacterium spp. etc.

III) Sulphur oxidizing microorganisms

Sulphur oxidizing bacteria oxidise sulphur containing compounds to sulphates which are utilized by plants. Also these bacteria convert sulphide into sulphuric acid, which act on insoluble soil compounds like Calcium carbonate, Magnesium carbonate, Calcium silicate, Tri-calcium phosphate and bring them in soluble state. These soluble compounds are then absorbed by the plants.

E.g. *Thiobacillus spp.*

IV) Organic matter decomposing microorganisms

The organic matter in soil consists of carbohydrates, proteins, lipids, and other materials. Organic matter makes up from 2 to 10 % most agriculturally important soils.

All organic matter in soil is derived from the remains of microorganisms, plants, animals, their waste products and the biochemical activities of various microorganisms. A great portion of organic matter is of plant origin mostly dead roots, wood, barks and leaves. A second source of organic matter is the vast number of bacteria, fungi, algae, protozoa and viruses, which can total billions per gram of fertile soil. These organisms break down organic substances producing and maintaining a continuous supply of inorganic substances that plants and other organisms require for growth. Much of the organic matter in soil is ultimately decomposed to inorganic substances such as ammonia, water, carbon dioxide and various compounds of nitrates, phosphates, and calcium. This conversion of organic compounds into simple inorganic compounds or into their constituent elements is called **mineralization.**

A considerable part of this organic matter in soil occurs as **Humus.** This dark material consists of partially decomposed organic matter, chiefly materials that are relatively resistant to decay.

The addition of organic matter either completely or partially decomposed is essential to the fertility of soil; moreover these spongy organic materials loosen the soil and thereby prevent the formation of **heavy crusts** and increase the pore spaces. The addition of pore spaces in turn increases aeration and water retention.

Production of biofertilizers

***The inoculum*:**

The inoculum is prepared from a stock culture preferably stored in sterile soil or on agar overlaid with oil. The organisms used in preparing the inoculum are preferably those able to form nitrogen-fixing nodules with several legumes (i.e., the so-called 'broad-spectrum' strains) / Azotobator species / Phosphate solubilizing / Sulphur oxidizing / Organic matter decomposing .

The inoculum added is preferably about 1% of the total volume and should have a density of 10^6-10^7 organisms per ml.

***Medium*:**

Most industrial media used consist of yeast extract or yeast hydrolysate, a carbohydrate source and mineral salts. In some media yeast extracts supply all the nitrogen, vitamins (especially biotin) and minerals required by the bacteria. Corn steep liquour and hydrolyzed casein are sometimes used to supplement yeast extract. In some media, one or more of potassium phosphate, magnesim sulfate, ferric chloride, and sodium chloride may be added. For the fast growers the carbohydrate source is usually sucrose; for the slow growers it may be mannitol, galactose or arabinose.

***Aeration*:**

In large fermentors air sparging without agitation is usually satisfactory.

***Time and temperature*:**

The temperature used is about 20°C and while fast growers attain high numbers (in excess of 140 x 10^9/ml) in about two to three days, slow growers in medium specially designed to facilitate their growth attain slightly less than this number in three to five days.

The fermentor:

Fermentors used for biofertilizer culture are small in comparison with those used for antibiotics. The larger sizes range from 1,000 to 2,000 liters.

Ordinarily they range from 5 liters through 40 liters to about 200 liters and are also usually quite unsophisticated compared with those used for producing antibiotics.

Packaging for use

After the fermentation of the organism it is packaged for delivery in either of two forms:
(a) as a coating on the seeds, (or seed inoculants) from which the bacteria develop at planting and invade the roots;
(b) direct application into soil with the seeds introduced shortly before or after soil inoculants.

Seed inoculants

Seed inoculants are more commonly used than soil inoculants. In seed inoculation the bacteria may be offered as a liquid or broth, frozen concentrates, freeze-dried or oil-dried preparation. No specific carrier is used for these preparations. Although gum Arabic, milk, and sucrose are sometimes added to these essentially liquid preparations, they still do not offer sufficient protection to the bacterial from the environment. The bacteria therefore die out quickly. By far the commonest preparations are offered with carriers.

A carrier is the material which binds the bacteria to the seed. Carriers should have a high water-holding capacity provided a nutritive medium for the growth of bacteria protect the bacteria from harmful environmental effect e.g. sunlight and favor their survival on the seeds and in the soil, and in particular they should not be toxic to the bacteria.

Agar may sometimes be used as a carrier but by far the most widely used carrier is peat. Other locally available materials may be used.

The use of peat as a carrier

Peat is the first stage in the formation of coal, which when freshly obtained is moist. It must be dried and milled or shredded. Peats vary in their properties and each must be studied and undesirable properties rectified before final use. For example peats mined in some parts of the world have a high

content of sodium chloride, which must be removed by leaching with water before the peat is used. Peats are also usually acid, and finely ground $CaCO_3$ up to about 5% is used to raise the pH to about 6.8.

The peat is therefore usually sterilized by hot air, steam (including autoclaving), gamma irradiation and chemical sterilants. Hot air and steam seem to be favored, as gamma-irradiation facilities are not always easily accessible and the post-treatment removal of chemical sterilants may sometimes be difficult.

Use of other carriers

Peat is not available in some parts of the world. Any carrier which meets the requirements indicated above would do. A wide range of materials have in fact been tried with success including lignite, coal, charcoal, bagasse, coir dust, composted straw plus charcoal, ground wheat straw, rice husk, and ground talc.

Biopesticide production

Insects are major pests of crops. Enormous losses occur when they attack various plant parts, often transmitting disease in the process. Even after harvest insects attack stored foods; this attack of stored foods is not limited to plant foods, but also includes animal foods such as dried fish. Besides the loss they cause in agriculture and food, insects are also vectors of various animal and human diseases.

In modern times insects have been controlled mainly with the use of chemicals. Over the past decade or so there has been a move away from the sole use of chemical control, and towards integrated control, which employs other methods as well as chemical control. The reasons for this include non-specificity of chemical insecticides leading to the destruction of pests as well as their natural predators, resistance to chemical insecticides, concern for the environment and human health since the insecticides enter drinking water from soil, and since some are toxic or carcinogenic. Finally due to increased cost of petroleum on which many of these insecticides are based, their cost has also increased.

Desirable Properties in Organisms to be Used for Biological Control

The following are desirable in microorganisms to be used in the biological control of insects:

a) The agent should be highly virulent for the target insect, but should kill no other insects.
b) The killing should be done quickly so that in the case of crops, damage is kept as low as possible, and in the case of vectors of disease before extensive transmission of the disease occurs.
c) The killing ability should be predictable.
d) The agent should not be harmful to man, animals or crops; in other words it should be safe to use.
e) It should be technically amenable to cheap industrial production.
f) When produced, it should be stable under the conditions of use such as under the high temperature and ultra violet light of ordinary sunlight.
g) It should be viable over reasonably long periods to permit storage and transportation as necessary.
h) It should ideally persist or recycle and/or be able to search for its host.

(i) *Bacteria*

A large number of bacteria are pathogenic to insects including *Bacillus* spp., *Pseudomonas* sp. *Klebsiella* sp., *Serratia marcescens.* In practice, spore formers have been developed commercially because they survive more easily in the environment than vegetative cells, but especially because they are amenable mass production. The four bacilli which have been produced for control purposes are:

(a) *Bacillus thuringiensis*: *B. thuringiensis* (commonly known as 'Bt') is an insecticidal bacterium, marketed worldwide for control of many important plant pests–mainly caterpillars of the Lepidoptera (butterflies and moths) but also mosquito larvae, and simuliid blackflies that vector river blindness in Africa. Bt products represent about 1% of the total 'agrochemical' market (fungicides, herbicides, and insecticides) across the world. The commercial Bt products are powders containing a mixture of dried spores and toxin crystals. They are applied to leaves or other environments where the insect larvae feed. The toxin genes have also been genetically engineered into several crop plants.

Bacilus thuringiensis produces at least three toxins, a Phospholipase C, a water-soluble heat stable B-exotoxin potentially toxic to mammals, and a crystalline, d-toxin or the parasporal body which is enclosed within the sporangium.

The crystalline d-toxin is the active principle against most insects. The spores and crystals are released into the medium in most strains of *B. thuringiensis* following the lysis of the sporangium.

Bacillus thuringiensis Insecticidal Toxin

B. thuringiensis strains produce two types of toxin. The main types are the **Cry** (crystal) toxins, encoded by different **cry genes**, and this is how different types of Bt are classified. The second types are the **Cyt** (cytolytic) toxins, which can augment the Cry toxins, enhancing the effectiveness of insect control. Over 50 of the genes that encode the Cry toxins have now been sequenced and enable the toxins to be assigned to more than 15 groups on the basis of sequence similarities.

Cry toxins are encoded by genes on plasmids of *B. thuringiensis*. There can be five or six different plasmids in a single Bt strain, and these plasmids can encode different toxin genes. The plasmids can be exchanged between Bt strains by a conjugation-like process, so there is a potentially wide variety of strains with different combinations of Cry toxins. In addition to this, Bt contains transposons (transposable genetic elements that flank genes and that can be excised from one part of the genome and inserted elsewhere). All these properties increase the variety of toxins produced naturally by Bt strains, and provide the basis for commercial companies to create genetically engineered strains with novel toxin combinations.

Mode of Action of Bt Toxin

The toxin of Bt is lodges in a large structure, the parasporal structure, which is produced during sporulation. The parasporal crystal is not the toxin. However, once it is solubulized a protoxin is released. The crystals are aggregates of a large protein (about 130-140 kDa) that is actually a **protoxin**, which must be activated before it has any effect. The crystal protein is highly insoluble in normal conditions, so it is entirely safe to humans, higher animals and most insects. However, it is solubilised in reducing conditions of high pH

(above about pH 9.5), the conditions commonly found in the mid-gut of lepidopteran larvae. For this reason, Bt is a highly specific insecticidal agent.

Once it has been solubilized in the insect gut, the protoxin is cleaved by a gut protease to produce an active toxin of about 60 kDa. This toxin is termed **delta-endotoxin**. It binds to the mid-gut epithelial cells, creating pores in the cell membranes and leading to equilibration of ions. As a result, the gut is rapidly immobilized, the epithelial cells lyse, the larva stops feeding, and the gut pH is lowered by equilibration with the blood pH. This lower pH enables the bacterial spores to germinate, and the bacterium can then invade the host, causing lethal septicaemia.

(b) *Bacillus moritai*:

This is used in Japan for the same purpose as *B. thuringiensis* serotypes H3 and H3A.

(c) *Bacillus popilliae*:

This is an obligate pathogen of the Japanese beetle *Popilla japonica* against which it is used. Since it is an obligate parasite it is produced in the larvae of the beetle.

(d) *Bacillus thuringinensis var. israelensis* (also known as serotype H14).

This was isolated in 1976 by Goldberg and Margalit from a mosquito breeding site in Israel. It has proved very effective in killing mosquito larvae and the black fly (*Simulium* spp). It has a (nearly 100%) kill of mosquito larvae and shows no adverse effect on non-target organisms. Its killing effect is therefore based principally on its crystalline delta-toxin, (d-toxin) which is resistant to both heat (surviving 80°C for 10 minutes and 60°C for 20 minutes) and ultra violet light.

(e) *Bacillus sphericus*:

Bacillus sphericus is a highly specific for mosquito larvae as *Bacillus thuringiensis*, var *israelensis (B.t.i.)*. However, whereas the lethality of *B.t.i.*

resides in toxic protein crystals formed during the spores of the organism, the toxin of *B. sphericus* resides in the cell wall of the organism. The toxin of *B. sphericus* works slowly (8-40 hours) compared with that of *B.t.i.* (2-10 hours). *Bacillus sphericus* however, has the advantage of being able to lay dormant in muds or sewage ponds and to recycle as susceptible mosquito larvae appear.

(ii) *Viruses*:

A large number of viruses have been isolated from insects. The advantages of viruses as biological control agents are that they are specific. Seven groups of insect pathogentic viruses have been identified. The most useful of them for biological control purposes are the **baculoviruses**, which are easily recognizable because the virus particles are included within a proteinaceous inclusion body large enough to be seen under a light microscope. (These inclusion bodies, polyhedrons and granules, are found in the nucleus of the host cell – hence they are nuclear polyhedrosis and granuloses).

The baculoviruses are the best candidates for insect control because they are

(a) effective in controlling insect populations, (b) restricted to a host range of invertebrates, (c) relatively easy to produce in large quantities and (d) stable under specific conditions because of the inclusion bodies.

Viruses are also very much effectively used as bioinsecticide. There are three major groups of viruses that can infect different insects.

These are:

(a) Nuclear polyhedrosis viruses (NPV),

(b) Granulosis viruses (GV) of Baculoviridae, and

(c) Cytoplasmic polyhedrosis viruses (CPV) of Reoviridae.

The NPVs are effective against moths and butterflies, while CPVs are effective mainly on caterpillars. These are used in different countries like USA, UK, Canada, lapan, Germany etc.

List if some viruses, registered trade names in parenthesis and target insects are given below:

i. NPV (ELCAR) — Tobacco budworm and Cotton bollworm.

ii. NPV (GYPCHEK) — Gypsy moth.

iii. NPV (VfROX) — European sawfly.

iv. CPV (MATSUKEMIN) — Pine caterpillar.

v. GV (MATEX) — Insects of different food crops like Codling moths.

Several experimental preparations are available and at least two (one each in the USA and Japan) have been produced on a commercial scale. The preparations are ingested when the insects consume leaves and other plant parts on which the virus particles have been sprayed. After ingestion the polyhedral inclusion bodies dissolve within the midgut; the released virions pass through the mid-gut epithelial cells into the haemocoel. Death of the larvae occurs four to nine days after ingestion.

(iii) *Fungi*:

All the four major groups of fungi, *Phycomycetes, Ascomycetes, Fungi Imperfecti* and *Basidiomycetes* contain members pathogenic to insects. The great difficulty with using fungi for biological control is that environmental conditions including temperature and humidity must be adequate for spore germination and insect cuticle penetration by the hyphae. Since these environmental conditions are not always assured the result is that fungi are used for biological control only in a few countries especially the USSR.

Fungi which have been most widely used as *Beauvaria bassiana* and *Metarrhizium anisopliae.* Others are *Hirsutella thompsonii Verticillium* and *Aschersonia aleyrodis. H.thompsonii* is being developed commercially as acaricide, for killing mites which attack plants, although a large number of other fungi attack mites. *H. thomposonii* has been found particularly active against mites which attack citrus. It is applied as the conidial powder and maximum effectiveness occurs at 27°C and under moist conditions or at relative humidities of 79-100%. *Coelomomyces* sp. is very effective against mosquitoes but its production is difficult because of the need of a secondary host. Most effective and specific against mosquitoes are *Culicinomyces* sp. which was isolated in Australia and produced a mortality rate on mosquitoes of 90-100%. *Tolypocladium cylindrosporum* is essentially like *Culicinomyces* in being highly specific for mosquitoes. *Lagenidium giganteum* and *Leptolegnia* sp have been shown to have high mortality for mosquitoes. All the above (except *Coelomomyces*) can be mass-produced by fermentation. *Beauvaria* and *Metarrhizium* already discussed have broad activity against mosquitoes.

(iv) *Protozoa*:

In contrast to the rapid action of viruses and spore-forming bacteria, killing by protozoa is slow and may take weeks. Furthermore they are difficult to produce, being accomplished only *in vivo.* Nevertheless they have been produced and successfully used experimentally for stored-product pests *(Matosia trogoderina)* mosquitoes *(Nosema algerae)* and grasshoppers *(Nosema pyrasta). Vavra vilivis* is also effective against mosquitoes and has properties similar to those of *Nogema algerae.* Studies sponsored by the WHO have shown that *N. algerae* does not seem to constitute a safety hazard for man. Factors favoring the use of *N. algerae* are sporelongevity, ease of spore-production under laboratory and especially cottage industry conditions and the probable impact on disease transmission by reducing the longevity of infected female mosquitoes. So far however protozoa have not been produced on an industrial scale for biological control.

PRODUCTION OF BIOLOGICAL INSECTICIDES

Microbiological insecticides are produced in one of three ways:

i) Submerged fermentation
ii) Surface or semi-solid fermentation
iii) *In vivo* production.

The first two are for facultative pathogens and the third is for obligate pathogens.

i) Submerged Fermentations

These have been used for the production of *Bacillus* spp. (excluding production of *B. poppillae* which is produced *in vivo*) and to a lesser extent, fungi.

***Medium*:**

In fermentation for *Bacillus thuringiensis* the active principle sought is the delta toxin found in the crystals. Media for submerged fermentation have been compounded by various workers in a number of patents. In one such

preparation, the initial growth in a shake flask occurred in nutrient broth; in the second shake flask, and in the seed fermentor beet molasses (1%), corn steep liquor (0.85%) and $CaCO_3$ (0.1%) were used.

A typical medium for production would be beet molasses (1.86%), pharmamedia (1.4%) and $CaCO_3$ (0.1%). Other production media contain corn starch (6.8%), sucrose (0.64%), casein (9.94%), corn steep liquor (4.7%), yeast extract (0.6%) and phosphate buffer (0.6%).

A third medium contained soya bean meal (15%), dextrose (5%), corn starch (5%), $MgSO_4$ (0.3%), $FeSO_4$ (0.02%), $ZnSO_4$ (0.02%) and $CaCO_3$ (1.0%).

The above media were used for agricultural strains of *B. thuringiensis* but could no doubt be used also for *B. thuringiensis var israelensis. Bacillus thuringiensis var insraelensis* and *Bacillus sphericus* do not require carbohydrates for growth and can grow well and produce materials which will kill the larvae of mosquitoes in a variety of proteinaceous materials such as commercial powders of soy products, dried milk products, blood and even materials from primary sewage tanks.

Effective powders of *B. sphericus* 1593 and *B. thuringiensis var israelensis* have been produced using discarded cow blood from abattoirs and various legumes.

***Extraction*:**

At the end of the fermentation, the active components of the broth are recovered by centrifugation, vacuum filtration with filter aid or by precipitation. Precipitation has been done with $CaCl_2$ and the acetone method yields products of very high potency. The fermentation beer may readily be diluted and used directly.

ii) Surface Culture

Surface culture techniques are used for fungi and for spore formers. The organisms after shake-flask growth are cultured in a seed tank from where the broth is transferred to flat bins with perforated bottoms. The semi-solid medium

is a mixture of an agricultural byproduct such as bran, an inert product such as kisselghur, soy bean meal, dextrose, and mineral salts. The use of this medium increases the surface area and hence aeration because of the thinness of its spread in the bins. Hot air is passed through the perforations to dry the material. It is ground, assayed and compounded to any required strength with inert material. Submerged, culture in which the hyphae are used have been carried out with good results in the United States using *Hirsutella thompsonii.*

***iii) In vivo* Culture**

In vivo culture methods are used for producing caterpillar viruses, mosquito protozoa and *Bacillus popillae.* The method is labor-intensive and could be easily applied for suitable candidates in developing countries where expertise for submerged culture production is usually lacking.

Once the organism has been obtained in a sufficient quantity to last for several years it is lyophilized and stored at low temperature. The viruses are introduced into the food of the larvae and the dead larvae are crushed, centrifuged to remove large particles and the rest are dried. The amount of viruses in each larva is variable but the virus content of between one and one hundred caterpillars should be sufficient to treat one acre in the case of cotton moths. Usually separate facilities are used for rearing the caterpillars, for infecting them and for the extraction of the virus particles. The preparation is then bioassayed and mixed with a suitable carrier.

FORMULATION AND USE OF BIOINSECTICIDES

Formulation of the bioinsecticides is extremely important. An insecticide shown to be highly potent under laboratory experimental conditions may prove valueless in the field unless the formulation has been correctly done. Since microorganisms cannot by themselves be patented, industrial firms producing bioinsecticides depend for their profits on the efficiency of their formulation (i.e., the inert material which ensures adequate presentation of the larvicide to the target insect). The inert material is referred to as a carrier or an extender. Carriers or extenders are the *solids* or *liquids* in which the active principle is diluted. When the carrier is a liquid and the active principle is suitable in it the application is a spray.

There are thus two types of formulation:

(a) powders and dusts

(b) flowable liquid;

Which of the two is manufactured depends to a large extent on the method of production and intended use of the insecticide.

Dusts

Semi-solid preparations based on waste plant products usually are compounded as dusts or powders because making them into liquid causes the bran to absorb water and prevent free flow thus leading to the clogging of conventional liquid applicators. The advantage of dusts is greater stability of the preparation. They are also useful when the insecticide is intended to reach the underside of low lying crops such as cabbages. Heavy rains unfortunately wash off dusts. They may also lead to inhalation of the bioinsecticides by the persons applying them. Diluents which have been used in commercial dust of *Bacillus thuringiensis* are celite, chalk, kaolin, bentonite, starch, and lactose. Lactose has also been used for diluting virus insecticide dusts. When the active principle is *absorbed* on to the extender (or filler), the extender is referred to as a *carrier*. If the extender or carrier is attractive to the insect as a food, oviposition site etc., then the extender or filler is known as a *bait*. Baits for *Bacillus thuringiensis* include ground corn meal, and for protozoa, cotton seed oil, honey, hydroxyethyl cellulose.

Liquid Formulation

Liquid formulations are usually made from water in which both the crystal and spores are stable. Sometimes oils and water/oil emulsions may be used. When liquids other than water are used it must be ascertained that they do not inactivate the active agent. Emulsifiers may be added to stabilize emulsions when these are used. Some emulsifiers which have been used for *B. thuringiensis* and viruses are Tween 80, Triton B 1956, and Span 20.

The nature of the surface on which the insecticide is applied and which may be oily, smooth or waxy may prevent the liquid from wetting the sprayed surface. *Spreaders* or wetting agents which are surface-tension reducers may be added. Wetting agents may be added to dusts to produce *wettable-powders* which are more easily suspended in water. Some wetting agents and spreaders which have been used for agricultural *Bacillus thuringienses* include alkyl

phenols Tween 20, Triton X114 and for viruses Triton X100 and Arlacel ‘C’ which are all commercial surface-tension reducing agents.

To prevent run-off of liquids or wettable powders, *stickers* or *adhesives* are added to hold the insecticide to the surface. Stickers which have been used for bacteria and viruses include skim milk, dried blood, corn syrup, casein, molasses, and polyvinyl chloride latexes.

Protectants are often added to insecticides which protect the active agent from the effect of ultra violet light, oxidation, desiccation, heat and other environmental factors which reduce the effectiveness of the active agent. These are usually trade secrets and their composition is not disclosed. Dyes combined with proteins such as brewers yeast plus charcoal, skin milk plus charcoal, and albumin plus charcoal have also proved effective in protecting virus preparations from the effect of the ultraviolet light of the sun. Microencapsulation of bioinsecticides with carbon also affords protection.

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