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T.Y. B.Sc. (Sem. VI) (CBCS)

MICROBIOLOGY

[601]: BIOPROCESS TECHNOLOGY

Unit 5
STUDIES OF SELECTIVE FERMENTATION PROCESS

Prepared By

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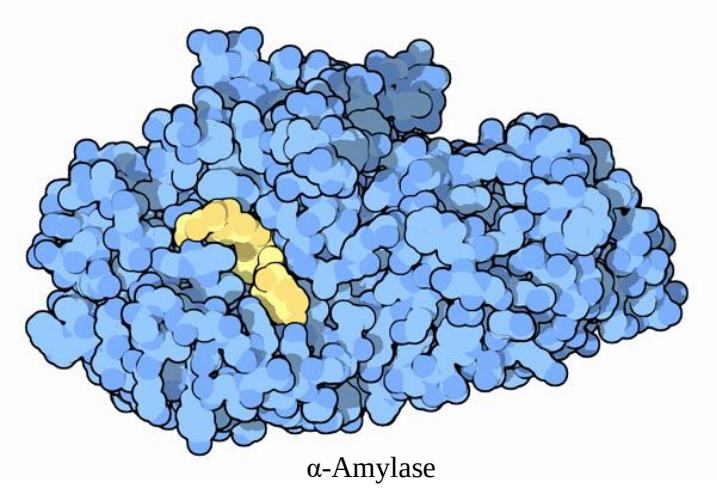
FERMENTATION PROCESS FOR AMYLASE PRODUCTION

Introduction:

An amylase is an enzyme that catalyzes the hydrolysis of starch into sugars. Amylase is present in the saliva of humans and some other mammals, where it begins the chemical process of digestion. Foods that contain large amounts of starch but little sugar, such as rice and potatoes, may acquire a slightly sweet taste as they are chewed because amylase degrades some of their starch into sugar.

Plants and some bacteria also produce amylase. Specific amylase proteins are designated by different Greek letters. All amylases are glycoside hydrolases and act on α-1,4-glycosidic bonds.

The α-amylases (EC 3.2.1.1) (alternative names: 1,4-α-D-glucan glucanohydrolase; glycogenase) are calcium metalloenzymes. By acting at random locations along the starch chain, α-amylase breaks down long-chain saccharides, ultimately yielding either maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. They belong to glycoside hydrolase family 13. Because it can act anywhere on the substrate, α-amylase tends to be faster-acting than β-amylase. In animals, it is a major digestive enzyme, and its optimum pH is 6.7–7.0.



α-Amylase

Microorganisms:

- For fungal α-amylase *Aspergillus niger* or *Aspergillus oryzae*
- For bacterial α-amylase *Bacillus subtilis* or *Bacillus amyloliquefacines* or *Bacillus licheniformis*

NOTE: The process of submerge culture method is only described here.

Raw material:

For fungal α-amylase:

- Carbon source: Corn starch 24 gm/L
- Nitrogen source: Corn steep liquor 36 gm/L
- Mineral**:** KCl 0.2 gm/L, Na_2HPO_4 47 gm/L, $CaCl_2$ 1 gm/L, $MgCl_2 \cdot 6H_2O$ 0.22 gm/L

Amylase biosynthesis is inhibited when there is glucose in the medium. The medium is steam sterilized. The sterilized medium is passed into a production fermenter for α-amylase production.

For bacterial α-amylase:

- Carbon source: Lactose 4.5 gm/L
- Nitrogen source: Ground soyabean meal 1.85 gm/L, Yeast extract 1.50 gm/L, Enzyme casein hydrolysate 0.65 gm/L
- Mineral: $MgSO_4 \cdot 7H_2O$ 0.04 gm/L
- Antifoam is required.

Inoculum preparation:

For bacterial α-amylase above mentioned any *Bacillus* can be selected as inoculum. Mutants which produce 250 times greater yields than the wild strain are preferred as inoculum.

Fermentation Process:

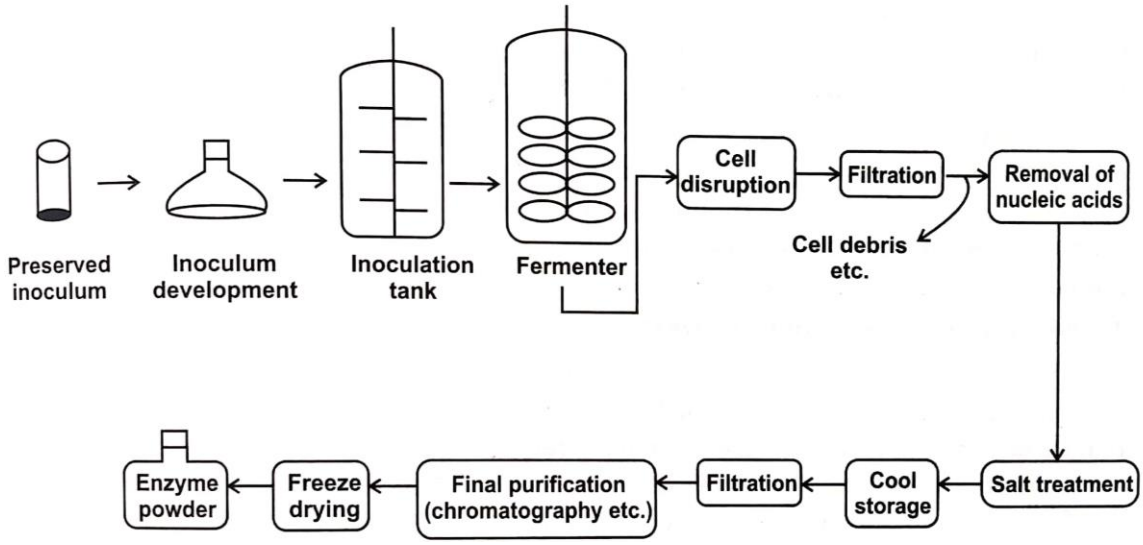
The type of fermenter that is used for fungal α-amylase is also used for bacterial α-amylase production.

For bacterial α-amylase,

- Fermentation is continued up to 4-6 days
- pH is maintained at 7.0.
- Calcium carbonate is used as buffer for maintaining neutral pH
- Temperature is maintained at 30-40 ˚C
- The production starts when the bacterial density reaches 10^9-10^{10} cells per ml.

For fungal α-amylase,

- Optimum pH is 7.0
- Calcium carbonate is used as buffer for maintaining neutral pH
- Temperature is generally maintained at 30-40 ˚C



An outline of the flowchart for the production of enzymes by microorganisms

Recovery:

Following steps are followed during the recovery of the enzyme after the completion of fermentation.

- In order to avoid denaturation of the enzyme, the fermentation broth is rapidly cooled at 5 ˚C and enzyme is extracted.
- Separation of cells is carried out by filtration of the refrigerated broth.
- The suspended particles present in the broth are removed with flocculating agents like calcium phosphate.
- Then enzyme is precipitated by using solvents like acetone or alcohol, or sometimes inorganic salts like ammonium sulphate or sodium sulphate.
- Sometime fractional precipitation of the enzyme is done to obtain it in purest form.

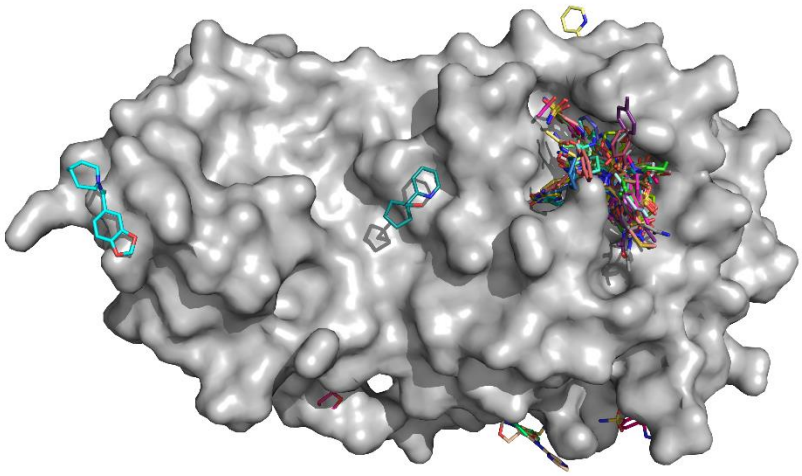
FERMENTATION PROCESS FOR PROTEASE PRODUCTION

Introduction:

A protease (also called a peptidase or proteinase) is an enzyme that catalyzes (increases the rate of) proteolysis, the breakdown of proteins into smaller polypeptides or single amino acids. They do this by cleaving the peptide bonds within proteins by hydrolysis, a reaction where water breaks bonds. Proteases are involved in many biological functions, including digestion of ingested proteins, protein catabolism (breakdown of old proteins), and cell signaling.

Without additional helping mechanisms, (such as an enzyme or other catalyst, to catalyze a reaction), proteolysis would be very slow, taking hundreds of years. Proteases can be found in all forms of life and viruses. They have independently evolved multiple times, and different classes of protease can perform (or "enable") (or "speed up") the same reaction by completely different catalytic mechanisms.

Proteases were first grouped into 84 families according to their evolutionary relationship in 1993, and classified under four catalytic types: serine, cysteine, aspartic, and metallo proteases. The threonine and glutamic-acid proteases were not described until 1995 and 2004 respectively. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the cysteine and threonine (proteases) or a water molecule (aspartic acid, metallo- and acid proteases) nucleophilic so that it can attack the peptide carboxyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine, or threonine as a nucleophile. This is not an evolutionary grouping, however, as the nucleophile types have evolved convergently in different superfamilies, and some superfamilies show divergent evolution to multiple different nucleophiles.



Protease

Microorganisms:

Proteases are commercially produced by both fungi and bacteria based on optimum pH for their activity.

By bacteria, such as species of *Bacillus, Pseudomonas, Clostridium, Proteus* and *Serratia.*

By fungi such as *Aspergillus niger, Aspergillus flavus, Aspergillus oryzae, Aspergillus wentii, Mucor delemar, Mucor miehei, Amylomyces rouxii, Penicillium roquefortii* etc.

Raw material:

For fungal proteases:

- Carbon source: Starch 40 gm/L
- Nitrogen source: Soyabean meal 30 gm/L, Ground barley 100 gm/L
- Mineral**:** $CaCO_3$ 5 gm/L,

For bacterial proteases:

- Carbon source: Starch hydrolysate 50 gm/L
- Nitrogen source: Soyabean meal 20 gm/L, Casein 20 gm/L
- Mineral: Na_2HPO_4 3.3 gm/L

Inoculum preparation:

There are two types of proteases;

1. Alkaline serine proteases
2. Acid proteases

Alkaline serine proteases are mainly produced by Bacillus licheniformis by submerge culture method. Acid proteases are mostly produced by fungi by either submerge culture method or semisolid culture method, depending on the fungal species.

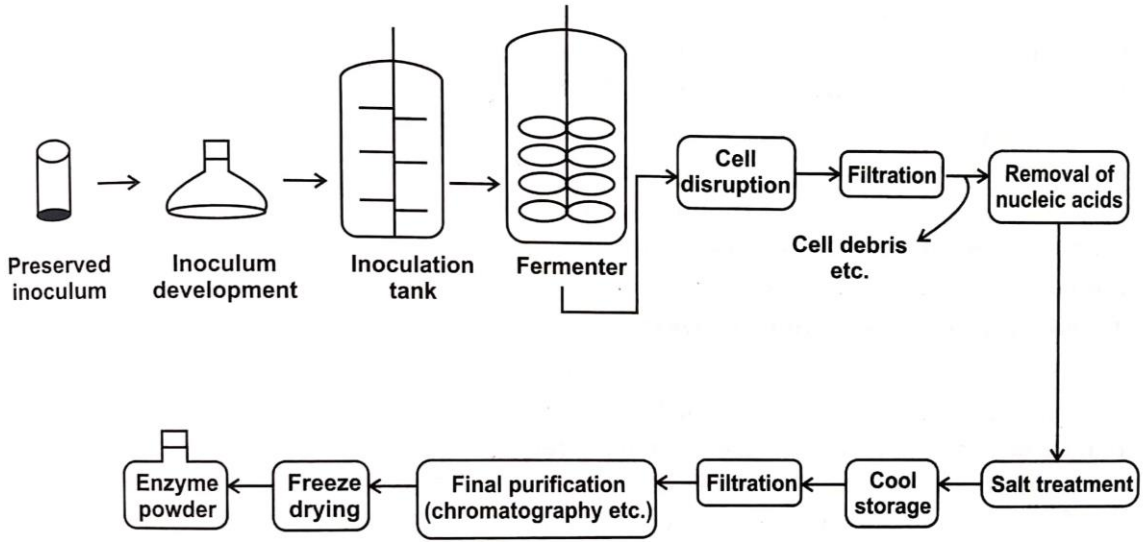
Fermentation Process:

For bacterial proteases, a high carbohydrate content medium is utilized to stimulate protease activity and depress amylase production, although the final product does contain some amylase activity.

- Fermentation is continued up to 3-5 days
- Temperature is maintained at 37˚C

For fungal proteases,

- Fermentation is continued up to 3 days
- Temperature is maintained at 30˚C
- *Mucor miehei* is used to produce acid proteases by submerged culture method, but requires 7 days for completion.



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- Sometime fractional precipitation of the enzyme is done to obtain it in purest form.

By product:

- Amylase

FERMENTATION PROCESS FOR ETHANOL (ETHYL ALCOHOL) PRODUCTION

Introduction:

Ethanol fermentation, also referred to as alcoholic fermentation, is a biological process in which sugars such as glucose, fructose, and sucrose are converted into cellular energy and thereby produces ethanol and carbon dioxide as metabolic waste products. Because yeasts perform this conversion in the absence of oxygen, ethanol fermentation is classified as anaerobic.

Ethanol fermentation occurs in the production of alcoholic beverages and ethanol fuel, and in the rising of bread dough. Fermentation process has virtually disappeared as result of development of chemical ones. Because, High prices of fermentable substrates and increasing economic competition from the petrochemical industry.

First alcohol was used for consumption. Then letter, demand for the universal solvent & small amount of industrial alcohol is mixed with motor fuels such as gasoline.

Microorganisms:

The choice of fermentation organism for industrial alcohol production depends to some extent, on the type of carbohydrate present in the medium.

Yeast: *Saccharomyces cerevisiae, Schizosaccharomyces pombe.*
Bacteria: *Zymomonas mobilis*
Fungi: *Funaria oxysporum*

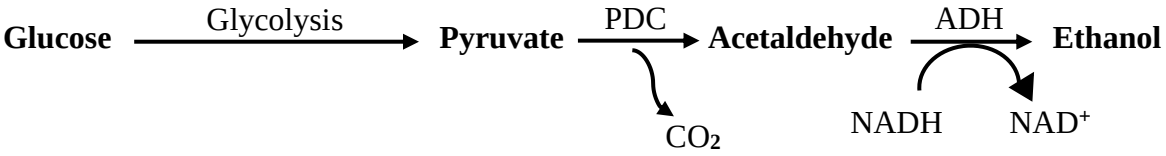
The strain of yeast is use because of following reason;

1. It should produce large quantity of alcohol.
2. Fast growing strain.
3. It should tolerate high amount of alcohol.
4. It has uniform and stable biochemical properties.

The different types of yeast, saccharomyces cerevisiae is the industrially important yeast for alcohol fermentation. Saccharomyces cerevisiae consists of different strains and varieties, which are used for different industrial applications. Yeast, which is used for ethanol production, is called "**Bakers strains**". Those used in beer making are called "**Brewing strains**" and wine making are called "**wine strains**". This differentiation is based on specific properties of yeast required by the particular industry.

Biosynthetic pathway:

Under aerobic condition *Saccharomyces cerevisiae* grow well but no alcohol production. Under anaerobic condition hoe ever growth slow and pyruvate from glycolytic pathway is split with pyruvate decarboxylase in to acetaldehyde and CO_2 then its reduction done with alcohol dehydrogenase and give ethanol.



PDC = Pyruvate decarboxylase
ADH = Alcohol dehydrogenase

Inoculum preparation:

- The Inoculum range from 8-10%.
- Rapid growth of yeast cell mass producing at optimum pH 4.5-5.0.
- The optimum temperature 28-30 ˚C.
- 10 ml of sterile medium in test tube is incubated with pure yeast culture for appropriate time with optimum temperature and pH.
- Then tube culture transfer to flask (200ml), then suitable glass container (4 L) then finally added to fermenter as per requirement fermentation process Inoculum will add.
- The operation of Inoculum to the fermentation medium is called pitching.

Raw material:

Verities of carbohydrates available as waste products of agricultural industry are use as fermentation medium.

- Saccharide materials – molasses, whey, fruit juices
- Starchy materials- potato, starch, cornstarch, oats.
- Cellulose material- sulphatic waste liquor, wood or wood waste product from processed wood.
- However Starchy material & cellulosic material whenever use pretreatment requires e.g. saccharification where starch get hydrolyzed and thus forming fermentable sugar maltose and glucose.

For satisfactory progress and compilation of the alcohol fermentation, the following condition are carefully controlled:

1) **“C” source:** Sugar concentration in the range 10-18% as satisfactory. Sugar con. 12% mostly used. e.g., cane molasses contains sucrose as carbon source. Sucrose content of this raw material is about 48-55%. Wort is prepared in such a way as contain about 10% sugars by adding water. High concentration affect yeast adversely while low con. Make process uneconomic.

2) **"N" source:** many organic and inorganic N- compounds may be used in this fermentation. Ammonium sulphate (0.15gm per 2.5 gallons) for growing yeast cells. Excess nitrogen is objectionable since it promotes the growth of yeast inhibiting the fermentation.

3) **pH:** Fermentation medium adjust to 4.8-5.0. Higher values of pH increase the chances of contamination while lower pH the yield of ethyl alcohol.

4) **Temperature:** 70-80 ˚F. The temperature can also control by cold water spray around the fermenter. Higher temperature favors the growth of yeast and also causes loss of alcohol due to evaporation.

Yield: on average 0.4 gallons of ethyl alcohol is obtain from one gallon of molasses. Strict technical condition is essential to ensure satisfactory yield.

Recovery:

- The fermented liquid is allowed to settle down for few hours and then distilled in analyzer & rectifier column to obtain rectified spirit (ethyl alcohol) and fused oil (Higher alcohol).
- Large distillers make use of patent stills (Coffey's still or llgis or barbets still) Coffey's still composed of two columns.
- The distilled analyzer at the top & follow zigzag course down the column. Steam enters at the bottom. The vapors (steam & alcohol) issuing from the top of the analyzer enter to the rectifier at the bottom.
- The rectifier is composed of a specifically designed fractioning column with a number of chambers.
- The less volatile constituents gradually condense & are drowned off from a higher point of the still.
- The head product which are very small consistent of aldehydes, formic esters etc. which owing to their greater volatility passes out through the top of the column, along with small quantity of uncondensed alcohol. The higher alcohol removes from the receiver every 2 or 3 days.

By product:

- Carbon dioxide
- Yeast
- Distillery effluent
- Liquefied Carbon dioxide use in soft drink industry.
- Yeast which has a higher content of protein, vitamin and essential elements.
- Alcohol product some quantities of higher alcohols (isoamyl alcohol) collectively called fused oil are also formed. After refining they are used in small quantities in the manufacturing of perfumes.

FERMENTATION PROCESS FOR CITRIC ACID PRODUCTION

Introduction:

Citric acid (C6H8O7) (2-hydroxy-propane-1,2,3-tricarboxylic acid) derives its name from the Latin word citrus, the citrus tree, the fruit of which resembles a lemon. The acid was first isolated from lemon juice. It is a natural preservative/conservative and is also used to add an acidic, or sour, taste to foods and soft drinks. In biochemistry, the conjugate base of citric acid, citrate, is important as an intermediate in the citric acid cycle.

It is used mainly as an acidifier, as a flavoring, and as a chelating agent. Citrate salts of various metals are used to deliver those minerals in a biologically available form in many dietary supplements. The buffering properties of citrates are used to control pH in household cleaners and pharmaceuticals. In Cosmetics, Citric acid is widely used as a pH adjusting agent in creams and gels. Citric acid can be used in food coloring.

Microorganisms:

The choice of fermentation organism for industrial citric acid production depends to some extent, on the type of carbohydrate present in the medium.

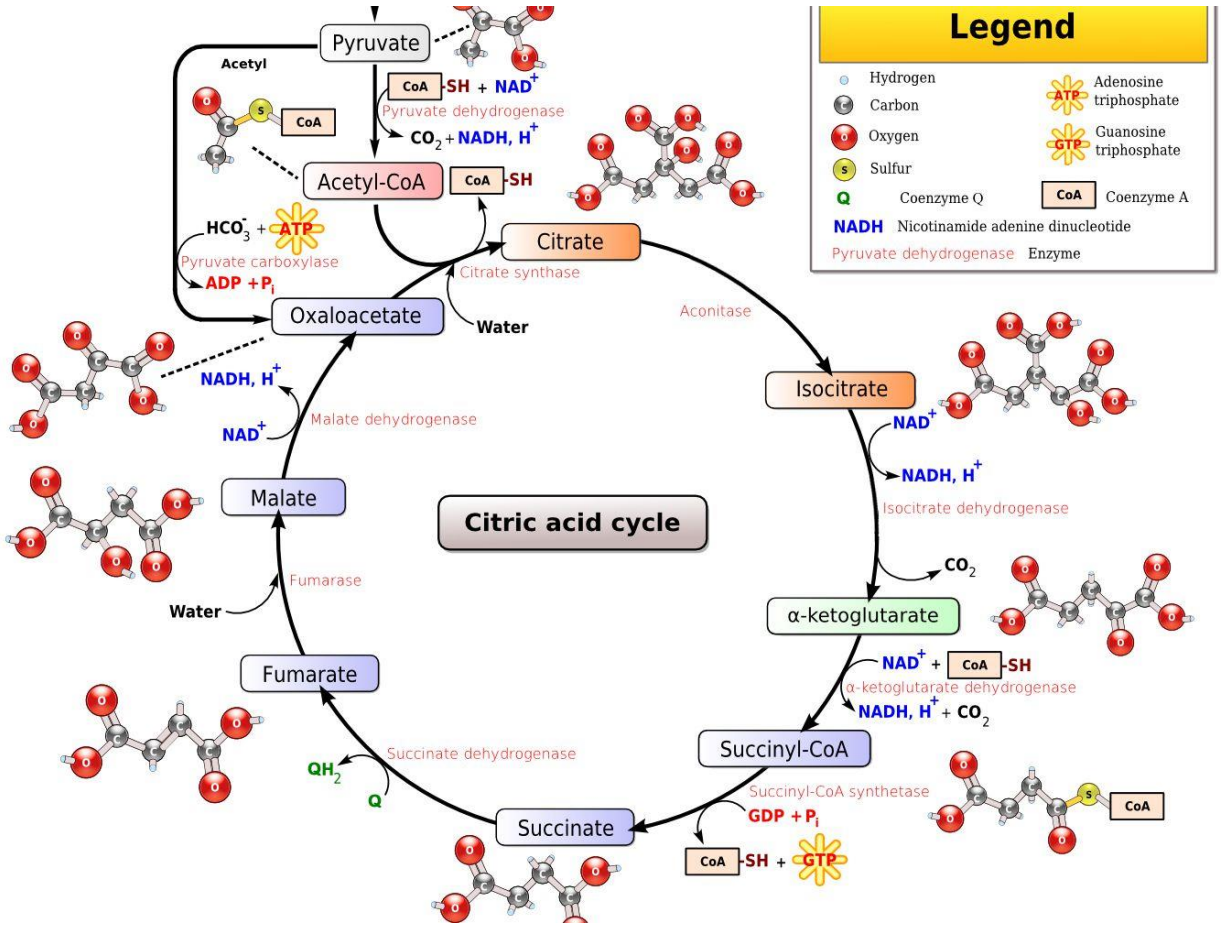
Fungi: *Aspergillus niger,* other species are *A. awamori, A. nidulans, A. fonsecaeus, A. luchensis, A. phoenicus, A. wentii, A. saitoi, A. flavus, Penicillium janthinellum, P. restrictum, P.botrytis).*

Yeast: *Candida species*

Bacteria: *Arthrobacter* and *Actinomycetes*

Biosynthetic pathway:

The citric acid cycle begins with the transfer of a two-carbon acetyl group from acetyl-CoA to the four-carbon acceptor compound (oxaloacetate) to form a six-carbon compound citric acid (citrate).



Fermentation medium:

Carbon source: The carbon source for the citric acid fermentation has been the focus of much study, frequently with a view to the utilizing polysaccharide sources. In general, only sugars that are rapidly taken up by the fungus. The carbon source used in industrial fermentations is typically beet molasses, although cane molasses, fruit pulp, polysaccharides and sugars are used. Production rates are usually achieved at 14-22% of sugar.

Nitrogen and phosphate limitation: Sources of nitrogen that have been used include urea and yeast/malt extract Complex media such as molasses are rich in nitrogen containing compounds and rarely need to be supplemented with a nitrogen source. The high purity media that are used mainly in research laboratories are generally supplemented with ammonium salts, particularly ammonium nitrate and ammonium sulfate, to provide the necessary nitrogen.

Broth pH: The pH of the medium is important, the pH for citric acid production needs to be low (pH≤2). Increasing the pH to 4.5 during the production phase reduces the final yield of citric acid by up to 80%.

Temperature: The fermentation is conducted at approximately 28 to 30˚C with proper aeration being of importance.

Trace elements: *A. Niger* requires certain trace metals for growth. The metals that must be limiting include. Zn, Mn, Fe, Cu, heavy metals and alkaline metals.

Fungal morphology: In submerged culture, the morphology of filamentous fungi varies between pellets and free filaments, depending on culture conditions and the genotype of the strain.

Preparation of Inoculum:

The materials used, as an inoculum for the production of citric acid is a spore suspension. Spores are produced in glass bottles, on solid substrate at 25˚C with incubation time of 10-14 days. A nutrient solution containing 15% sugar (from molasses) is used in the seed fermented and to induce the formation of mycelium in the form of pellet, cyanide ions are added. The spore germinates at 32˚C & form pellets 0.2-0.5mm in diameter within 24 hours. During this period, the pH falls to 4.3. These pellets are then used as the inoculums for the production fermenter. In some cases, production fermenter is inoculated directly with spores.

Fermentation of citric acid:

Citric acid fermentation presently is conducted on industrial scale by two methods:

1). Sub-merged aerated fermentation.

2). Stationary tray fermentation.

SUB-MERGED AERATED FERMENTATION: Eighty percent of the world's supply of citric acid is produced by submerged processes. Three factors are especially important for production in submerged process:

a). Quality of the material used to construct the fermenter: Fermenter for citric acid production must either be protected from acids or constructed of stainless steel with plastic liners, because at pH value between1-2 the heavy metal leaches.

b). Mycelium structure: If the mycelium is loose and filamentous, with limited branches and no chlamydospores, little citric acid is produced. Thus, mycelium for optimal production rates consists of very small

c). Oxygen supply: Although *Aspergillus niger* requires relatively little oxygen, it is sensitive to oxygen deficiency. Short interruptions in the oxygen supply cause production to cease irreversibly.

STATIONARY TRAY FERMENTATION: Stationary tray fermentation utilizes stationary pans or trays containing a shallow layer of medium (1 to 2.5mm in depth). The medium is inoculated by blowing spores across the surface of a shallow layer medium, so that they float, and that air is blown gently over the resulting mycelial mat. The trays are harvested after approximately 7-10 days of incubation, with the citric acid yield reported to be on order of 60-80 gm. (Or greater) of anhydrous citric acid per 100 grams sugar.

YIELD: Reported yields from laboratory and pilot plants are in the range of 70-90% conversion of citric acid when Aspergillus niger is incubated on media rich in carbohydrates.

RECOVERY:

- The citric acid is difficult to recover from the harvested fermentation broth and the recovery is further complicated by the presence of unfermented sugars, other acid fermentation products & inorganic impurities.
- Rotating filters or centrifuge is used to separate the mycelium and if oxalic acid is formed as a side product, it is precipitated as calcium oxalate at low pH., thus leaving behind the citric in the solution in the form of monocalcium citrate.
- At pH 7.2 and 70-90˚C, citric acid is precipitated which can in turn be separated by means of rotating filter and dried. For some uses critic acid is further purified by adding sulphuric acid to dissolve citric acid and forming precipitate of calcium sulfate.
- The subsequent recovery steps include treatment with activated carbon, treatment with cat ion and anion exchangers and crystallization of citric acid or citric acid monohydrate. At 40˚C citric acid crystallizes as the anhydrous acid and below 36.5˚C as the monohydrate. They require purity of the product depends on its intended use. Citric acid for addition to food must of course be pure than needed for industrial purposes.

FERMENTATION PROCESS FOR RIBOFLAVIN (VITAMIN B_2) PRODUCTION

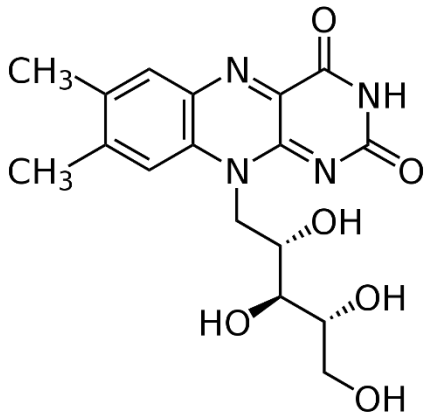
Introduction:

Riboflavin, also known as vitamin B_2, is a vitamin found in food and used as a dietary supplement. It is required by the body for cellular respiration. Food sources include eggs, green vegetables, milk and other dairy products, meat, mushrooms, and almonds. Some countries require its addition to grains.

As a supplement it is used to prevent and treat riboflavin deficiency. At amounts far in excess of what is needed to meet dietary needs as a nutrient, riboflavin may prevent migraines. Riboflavin may be given by mouth or injection. It is nearly always well tolerated. Normal doses are safe during pregnancy. Riboflavin was discovered in 1920, isolated in 1933, and first synthesized in 1935. It is on the World Health Organization's List of Essential Medicines.

According to IUPAC rules, riboflavin [83-88-5] is called 7,8- dimethyl-10-(d-1'-ribityl) isoalloxazine, also known as vitamin B2 or lactoflavin. The daily human demand for riboflavin is around 1.7 mg, and deficiencies lead to various symptoms such as, e.g., versions of dermatitis. The vitamin cannot be stored in the body and a constant intake is required.

Chemical synthesis was the first production method to be established and is still dominating, but in recent years the production is shifting more and more to fermentation. Green plants, most bacteria, and molds, however, can produce their own riboflavin.



Riboflavin

Microorganisms:

At present, three organisms are used for the industrial production of riboflavin by fermentation:
The filamentous fungus *Ashbya gossypii* (BASF, Germany).
The yeast *Candida famata* (ADM, USA)
A genetically engineered strain of *Bacillus subtilis* (DSM, Germany).

Biosynthetic pathway:

The biosynthesis of one riboflavin molecule requires one molecule of GTP and two molecules of ribulose 5-phosphate as substrates.

The imidazole ring of GTP is hydrolytically opened, yielding a 4, 5-diaminopyrimidine which is converted to 5-amino-6-ribitylamino-2, 4(1H,3H)-pyrimidinedione by a sequence of deamination, side chain reduction and dephosphorylation.

Condensation of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione with 3, 4-dihydroxy-2-butanone 4-phosphate obtained from ribulose 5-phosphate affords 6,7-dimethyl-8-ribityllumazine.

Dismutation of the lumazine derivative (riboflavin synthase) yields riboflavin and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione, which is recycled in the biosynthetic pathway.

Media Preparation:

Carbon source: Glucose, Sunflower oil

Nitrogen source: Peptone, Malt extract, Yeast extract, Methionine

Minerals: $MgSO_4 \cdot 7H_2O$, K_2HPO_4

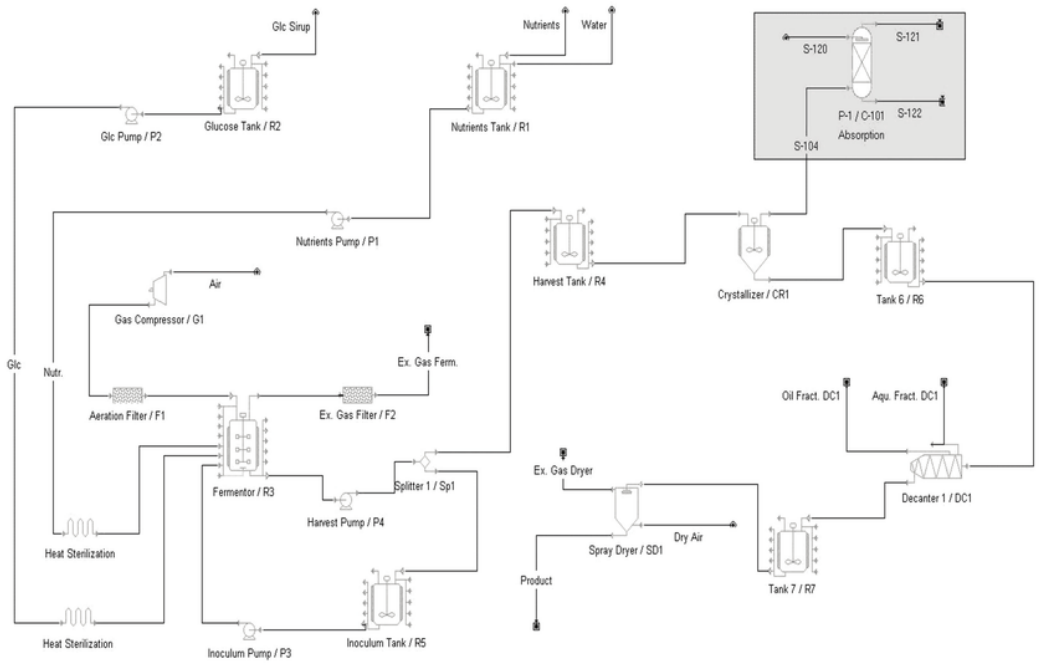
The medium's composition does not allow sterilization of all components mixed together and using classical batch conditions (121◦C, 20 minutes). Therefore, the medium would be divided into several groups:

- Glucose (70%) and sunflower oil
- peptone, yeast and malt extracts
- salts in water
- methionine

The latter is sterilized by filtration. Sulfuric acid does not require sterilization.

Only two separate solutions have to be prepared:

1. 70% glucose
2. other nutrients



Inoculum Preparation:

- In several steps the necessary seed cultures are prepared in different seed fermenters. The last seed culture is the start inoculum for the main fermentation.
- The duration of a seed-fermentation is around 50 hours, while the main fermentation lasts about 500 hours.
- Fermentation requires aeration accomplished by a gas compressor and a sterile filter.
- Exhaust gases are filtered by a second filter.
- A small fraction of the harvested broth is put into another tank and is used as inoculum for the next batch.

Yield: During this time the strain produces 27 g/L riboflavin.

Recovery:

- After fermentation the broth is harvested into the harvest tank.
- Part of the product crystallizes in the fermenter and also in the harvesting tank. Crystallization is completed in the crystallizer by evaporation of some of the water.
- Afterwards the suspension is stored in tank.
- From the decanter three streams are harvested, two liquid phases and the cell/crystal suspension. To achieve higher purity, a washing step is used with a second separation.
- The last step is drying, either using a spray dryer to obtain a powdered product or applying a spray granulation to obtain granulate.

FERMENTATION PROCESS FOR LYSINE PRODUCTION

Introduction:

Out of the twenty naturally occurring amino acids, L-Lysine ($C_6H_{14}N_2O_2$; MW 146.19) is one of the 9 essential (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) and commercially important amino acids, ecumenically found in naturally occurring proteins of all living organisms.

Its major commercial form is L-Lysine-HCl (L-Lysine monohydrochloride).

Signs & symptoms of Lysine deficiency:

Unintended weight loss, fatigue, RBC level unusually low, dizziness, headache, hair loss etc.

L-lysine is useful as medicament, chemical agent, food material (food industry) and feed additive (animal food).

L-lysine producing strains:

L-lysine producing strains of the gram-positive *Corynebacterium*, especially *Corynebacterium glutamicum, Brevibacterium flavum* and *Brevibacterium lactofermentum*, have been used for the last fifty years for the industrial production of amino acids.

Commercial production of L-lysine. Selected mutant strains with improved productivity characteristics are used today in industry for the production of L-lysine, which are obtained by various methods including classical mutagenesis light irradiation (UV), X-ray irradiation, radiation irradiation, plasma fusion, genetic engineering and any other techniques commonly used for mutation of microorganisms.

Biosynthesis of L-lysine:

Two pathways have been identified in nature for the synthesis of lysine. The diaminopimelate (DAP) pathway belongs to the aspartate derived biosynthetic family, which is also involved in the synthesis of threonine, methionine and isoleucine. Whereas the α-aminoadipate (AAA) pathway is part of the glutamate biosynthetic family.

The DAP pathway is found in both prokaryotes and plants and begins with the dihydrodipicolinate synthase (DHDPS) (E.C 4.3.3.7) catalyzed condensation reaction between the aspartate derived, L-aspartate semialdehyde, and pyruvate to form (4S)-4-hydroxy-2,3,4,5-tetrahydro-(2S)-dipicolinic acid (HTPA). The product is then reduced by dihydrodipicolinate reductase (DHDPR) (E.C 1.3.1.26), with NAD(P)H as a proton donor, to yield 2,3,4,5-tetrahydrodipicolinate (THDP). From this point on, four pathway variations have been found, namely the acetylase, aminotransferase, dehydrogenase, and succinylase pathways. Both the acetylase and succinylase variant pathways use four enzyme catalyzed steps, the aminotransferase pathway uses two enzymes, and the dehydrogenase pathway uses a single enzyme. These four variant pathways converge at the formation of the penultimate product, meso-diaminopimelate, which is subsequently enzymatically decarboxylated in an irreversible reaction catalyzed by diaminopimelate decarboxylase (DAPDC) (E.C 4.1.1.20) to produce L-lysine.

The DAP pathway is regulated at multiple levels, including upstream at the enzymes involved in aspartate processing as well as at the initial DHDPS catalyzed condensation step. Lysine imparts a strong negative feedback loop on these enzymes and, subsequently, regulates the entire pathway.

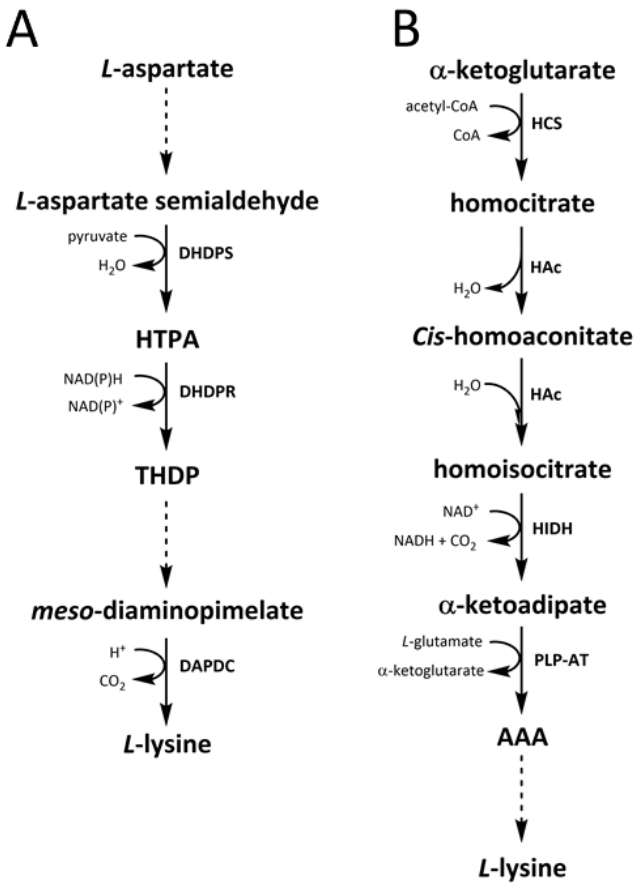
The AAA pathway involves the condensation of α-ketoglutarate and acetyl-CoA via the intermediate AAA for the synthesis of L-lysine. This pathway has been shown to be present in several yeast species, as well as protists and higher fungi. It has also been reported that an alternative variant of the AAA route has been found in *Thermus thermophilus* and *Pyrococcus horikoshii*, which could indicate that this pathway is more widely spread in prokaryotes than originally proposed.

The first and rate-limiting step in the AAA pathway is the condensation reaction between acetyl-CoA and α-ketoglutarate catalyzed by homocitrate-synthase (HCS) (E.C 2.3.3.14) to give the intermediate homocitryl-CoA, which is hydrolyzed by the same enzyme to produce homocitrate. Homocitrate is enzymatically dehydrated by homoaconitase (HAc) (E.C 4.2.1.36) to yield cis-homoaconitate. HAc then catalyzes a second reaction in which cis-homoaconitate undergoes rehydration to produce homoisocitrate. The resulting product undergoes an oxidative decarboxylation by homoisocitrate dehydrogenase (HIDH) (E.C 1.1.1.87) to yield α-ketoadipate. AAA is then formed via a pyridoxal 5′-phosphate (PLP)-dependent aminotransferase (PLP-AT) (E.C 2.6.1.39), using glutamate as the amino donor. From this point on, the AAA pathway varies with on the kingdom.

In fungi, AAA is reduced to α-aminoadipate-semialdehyde via AAA reductase (E.C 1.2.1.95) in a unique process involving both adenylation and reduction that is activated by a phosphopantetheinyl transferase (E.C 2.7.8.7). Once the semialdehyde is formed, saccharopine reductase (E.C 1.5.1.10) catalyzes a condensation reaction with glutamate and NAD(P)H, as a proton donor, and the imine is reduced to produce the penultimate product, saccharopine. The final step of the pathway in fungi involves the saccharopine dehydrogenase (SDH) (E.C 1.5.1.8) catalyzed oxidative deamination of saccharopine, resulting in L-lysine.

In a variant AAA pathway found in some prokaryotes, AAA is first converted to N-acetyl-α-aminoadipate, which is phosphorylated and then reductively dephosphorylated to the ε-aldehyde. The aldehyde is then transaminated to N-acetyl-lysine, which is deacetylated to give L-lysine. However, the enzymes involved in this variant pathway need further validation.

NOTE: These pathways are just for information. There is total 2 different pathways for synthesis of Lysine. No need to remember whole pathways, just read and understand image given below.



Pathway regulation:

Pathway is regulated at two points:

- First through feedback inhibition of aspartate kinase by the levels of both Threonine and L-lysin.
- Second through the control of the level of dihydrodipicolinate synthase. Increased production of L-lysine may be therefore obtained in Corynebacterium by deregulating, desensitizing feedback inhibition by L-lysine and L-threonine and by increasing the activity of these two enzymes.

Fermentation medium:

Carbon source: Various carbohydrates are utilized individually or as a mixture for the production of L-lysine such as glucose, fructose, sucrose, blackstrap molasses, lactose, maltose, starch and cellulose, cellulose hydrolysate, organic acids such as acetic acid, propionic acid, benzoic acid, formic acid, malic acid, citric acid and fumaric acid, alcohols such as ethanol, propanol, inositol and glycerol and certainly hydrocarbons, oils and fats such as soy bean oil, sunflower oil, groundnut oil and coconut oil as well as fatty acids such as e.g. palmitic acid, stearic acid and linoleic acid.

Those substances may be used individually or as mixtures.

Nitrogen source: Various sources of nitrogen are utilized individually or as mixtures for the commercial and pilot scale production of L-lysine, including inorganic compounds such as gaseous and aqueous ammonia, ammonium salts of inorganic or organic acids such as ammonium sulfate, ammonium nitrate, ammonium phosphate, ammonium chloride, ammonium acetate and ammonium carbonate.

Alternatively, natural nitrogen containing organic materials like soybean-hydrolysate, Soybean meal, corn steep liquor, yeast extract, meat extract, malt extract, urea, peptones and amino acids may also be utilized.

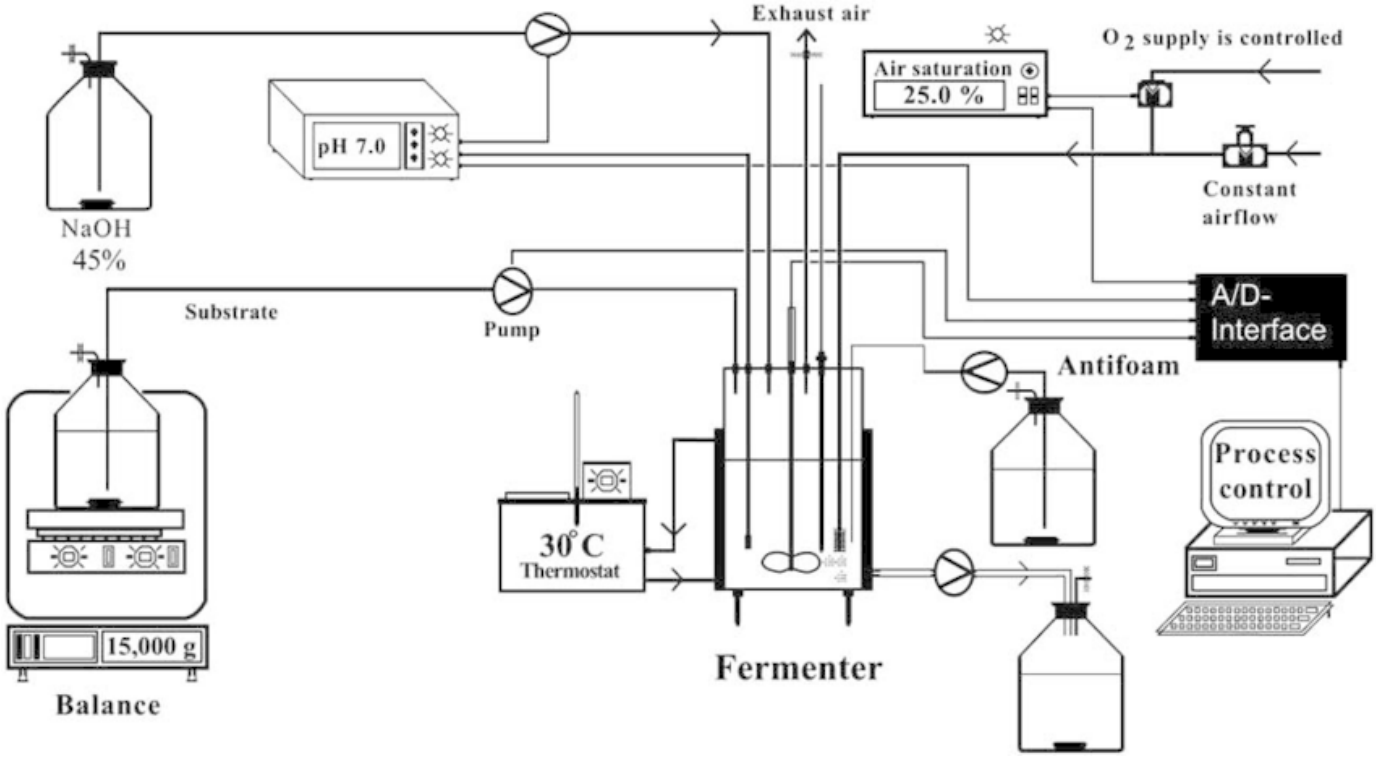
Inorganic salts and growth factors: Inorganic salts of various metals, like magnesium (e.g., magnesium sulfate), calcium, potassium, sodium, iron (e.g., iron sulfate), manganese, and zinc or traces of other metals. Phosphoric acid, potassium dihydrogen phosphate (KH_2PO_4) or dipotassium hydrogen phosphate (K_2HPO_4) or the corresponding sodium salts are commonly used as source of phosphorus for the production of L-lysine.

Essential growth factors such as amino acids and vitamins (e.g., vitamin B1).

Antifoaming: Foaming occurring during fermentation is controlled by the addition of antifoams such as fatty acid polyglycol esters or silicone and polypropylene.

Temperature: Preferably 25-40˚C.

pH: pH ranging from 5 to 9.



L-Lysine purification:

- L-Lysine is separated and purified by suitable downstream processes involving classical separation or extraction methods.
- Cell separation - ultrafiltration or centrifugation, evaporation
- L-lysine culture broth subjected to ion exchange extraction, evaporation & crystallization, Freeze drying then decolorize with charcoal and is sold as a powder.
- Alternatively, spray dried pellets or liquid fermentation broth can be used as animal feed supplement.

FERMENTATION PROCESS FOR PENICILLIN PRODUCTION

Introduction:

The discovery of penicillin and its medicinal uses was the most important scientific discovery of the 20th century. Antibiotics are chemicals, effective at very low concentrations, created as part of the life process of one organism, which can kill or stop the growth of a disease-causing microbe.

In 1929, Alexander Fleming, a doctor and researcher at St. Mary's Hospital in London, England, published a paper on a chemical he called "penicillin", which he had isolated from a mold, *Penicillium notatum* which inhibit the growth of *Staphylococcus aureus*. Penicillin had prevented the growth of a neighboring colony of germs in the same Petri dish. Dr. Fleming was never able to purify his samples of penicillin, but he became the first person to publish the news of its germ-killing power.

Mode of Action:

Penicillin kills by preventing some bacteria from forming new cell walls. Bacteria reproduce by dividing to produce two new cells. They enlarge to about twice their size before the DNA chromosome is copied. The two new chromosomes move apart and a cell wall forms between them. But if penicillin is present, the new cell wall won't be able to form.

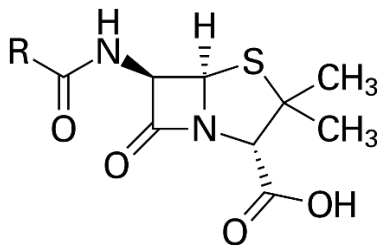
It doesn't harm old bacterial cell walls, but it stops new ones forming. This means the bacteria can't reproduce, so the disease can't spread.

Penicillin acts by blocking the activity of the enzyme transpeptidase, which cross connects long polymers of sugars that form the bacterial cell wall. The β-lactam ring on penicillin irreversibly blocks the activity of the enzyme by covalently bonding with the functional end of the enzyme.

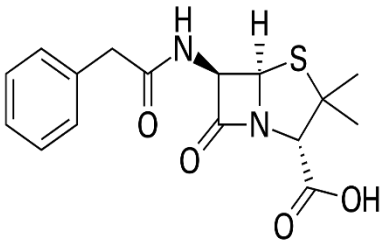
Categories of Penicillin:

There are two different categories of penicillin.

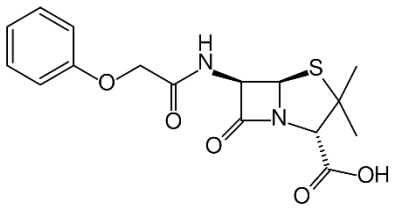
- Biosynthetic penicillin is natural penicillin that is harvested from the mold itself through fermentation.
- The other form of penicillin is known as semi-synthetic. There are all kinds of what are called semisynthetic derivatives of penicillin - like Ampicillin, Penicillin V, Carbenicillin Oxacillin, Methicillin, etc.



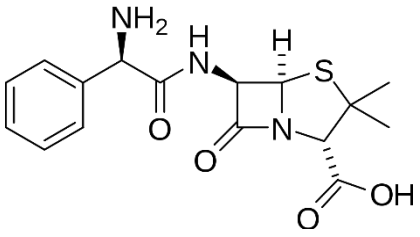
Penicillin



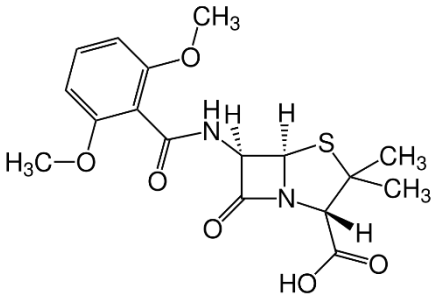
Penicillin G



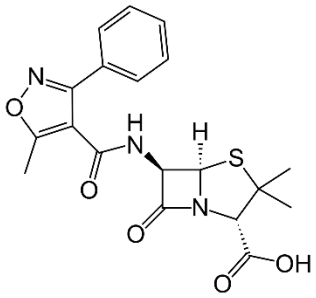
Penicillin V



Ampicillin



Methicillin



Oxacillin

These compounds consist of the basic Penicillin structure, but have been purposefully modified chemically by removing the acyl group to leave 6-aminopenicillanic acid and then adding acyl groups that produce new properties.

These modern semi-synthetic penicillins have various specific properties such as resistance to stomach acids so that they can be taken orally. Penicillin G is not stable in the presence of acid. Since our stomach has a lot of hydrochloric acid in it (the pH can be around 2.0), if we were to ingest penicillin G, the compound would be destroyed in our stomach before it could be absorbed into the bloodstream, and would therefore not be any good to us as a treatment for infection somewhere in our body.

It is for this reason that penicillin G must be taken by intramuscular injection - to get the compound in our bloodstream, which is not acidic at all. Many of the semisynthetic penicillin can be taken orally.

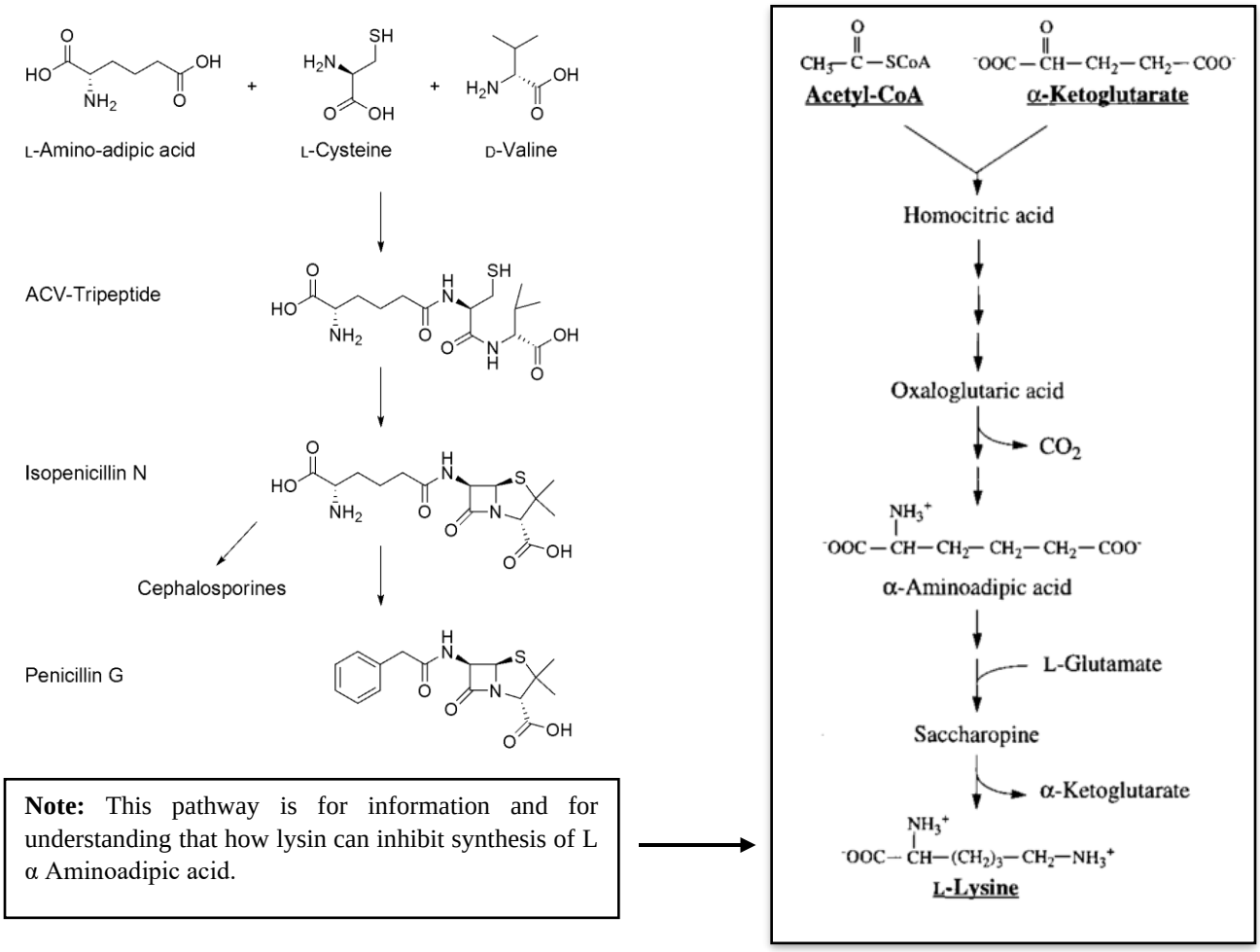
Biosynthesis:

Overall, there are three main and important steps to the biosynthesis of penicillin G (benzylpenicillin).

The first step is the condensation of three amino acids—L-α-aminoadipic acid, L-cysteine, L-valine into a tripeptide. Before condensing into the tripeptide, the amino acid L-valine must undergo epimerization to become D-valine. The condensed tripeptide is named δ- (L-α-aminoadipyl)-L-cysteine-D-valine (ACV). The condensation reaction and epimerization are both catalyzed by the enzyme δ-(L-α-aminoadipyl)-L-cysteine-D-valine synthetase (ACVS), a nonribosomal peptide synthetase or NRPS.

The second step in the biosynthesis of penicillin G is the oxidative conversion of linear ACV into the bicyclic intermediate isopenicillin N by isopenicillin N synthase (IPNS), which is encoded by the gene *pcbC*. Isopenicillin N is a very weak intermediate, because it does not show strong antibiotic activity.

The final step is a transamidation by isopenicillin N N-acyltransferase, in which the α-aminoadipyl side-chain of isopenicillin N is removed and exchanged for a phenylacetyl side-chain. This reaction is encoded by the gene *penDE*, which is unique in the process of obtaining penicillins.



Note: This pathway is for information and for understanding that how lysin can inhibit synthesis of L α Aminoadipic acid.

Regulation of penicillin biosynthesis:

Lysine inhibits penicillin biosynthesis because it is feedback inhibition of homocitrate synthetase, enzyme involved in L-α AAA synthesis.

Penicillin biosynthesis also affected by phosphate concentration & also show distinct catabolic repression by glucose in addition to regulation by concentration of ammonium ion, letter by an unexplained mechanism.

Raw material:

The medium should be favoring the growth of organism.

Carbon sources: Lactose (6%) give satisfactory result. Other than glucose Carbon source are also use Sucrose, Dextran, Starch & maltose.

Nitrogen sources: Corn Steep liquor, (Commercial N-source Pharma media can use). Additional N- source such as soya meal, yeast extract or whey can use.

Mineral Source: Mg, K, P, Zn, S, Cu helps proper growth & production.

Precursor: It can be added depending upon the penicillin type it differ.

Temperature:

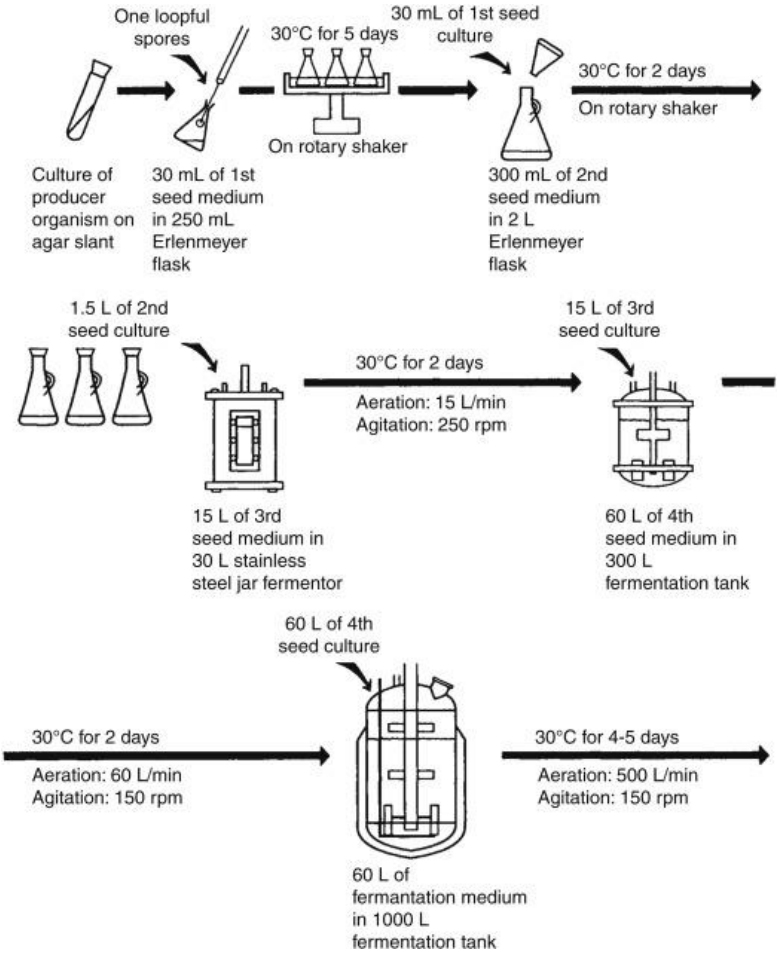
25-27 ˚C depending of strain.

Inoculum preparation:

It is stored using lyophilized spore. Because of great variability of yielding strain. Spore concentration: $5x10^6$/ml (Depend on production level.)

Once the overall conditions for growth have been established and there is a viable vegetative culture active inside the flask, it will be transferred to a 1 or 2 L bench- top reactor. This reactor will be fitted with a number of instruments to allow the culture to be better observed than it was in the shake flask. Typical parameters observed include pH, temperature, stirrer speed and dissolved oxygen concentration.

Once this has been successful the process is scaled up again to a pilot-scale bioreactor. This reactor will be similar in design to the bench-top reactor except it will have a size of about 100-1000 liters.



Production:

Penicillin G & V produce using submerged process in 40,000-200,000L fermenter. Due to difficulties with the oxygen supply, large tanks not be employed.

It is aerobic processes with a volume of oxygen supply 0.4-0.8mM/l/min. The required aeration rate is between 0.5-1.0 VVM (volume of air /volume of medium/min) depending of strain. Various turbine impellers are used for mixing.

Extraction & Purification:

- Divided in following steps:
- Removal of mycelium: Penicillin fermentation is filtered on a rotator vacuum filter use to remove mycelium & other solid material.
- Counter current solvent extractor: Phosphoric or sulfuric acid are added to lower the pH (2-2.5) in order to convert the penicillin to the anion form and the broth is immediately extracted in a Podbielniak counter current solvent extractor, with an organic solvent such as amyl acetate, methyl isobutyl ketone or butyl acetate.
- It is back extracted in to water from organic solvent by adding enough potassium or sodium hydroxide to form salt of penicillin.
- The resulting aqueous solution is again acidified of the penicillin & reextracted with methyl isobutyl ketone.
- Crystalline penicillin salt then washes and dried and the fine product must pass as Government standard.

FERMENTATION PROCESS FOR STREPTOMYCIN PRODUCTION

Introduction:

Streptomycin, produced by *Streptomyces griseus* is active against Gram (-) ve bacteria and against tuberculosis bacterium, *Mycobacterium tuberculosis*. However, it proved to be useful in the treatment of infections caused by Gram (+) ve especially resistant to penicillin. It is also useful in the control of plant diseases caused by bacteria as it acts systemically in plants.

One of the disadvantages of streptomycin is its neurotoxicity due to which hearing impairment and balance maintenance is lost in man due to prolonged streptomycin treatment at high dosage. Its reduction to dihydrostreptomycin results in the decreased toxicity.

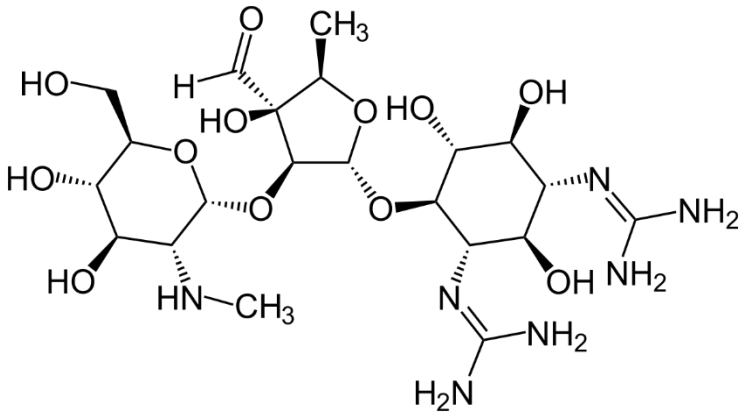
For this reason, in recent times only dihydrostreptomycin is being produced due to ready development of resistance against streptomycin. It is used mostly in conjunction with para aminosalicyclic acid or isoniazid (isonicotinic acid hydrazide) which minimizes resistance build up in sensitive microorganisms.

Mode of Action:

Streptomycin is a protein synthesis inhibitor. It binds to the small 16S rRNA of the 30S subunit of the bacterial ribosome irreversibly, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit. This leads to codon misreading, eventual inhibition of protein synthesis and ultimately death of microbial cells through mechanisms that are still not understood.

Speculation on this mechanism indicates that the binding of the molecule to the 30S subunit interferes with 50S subunit association with the mRNA strand. This results in an unstable ribosomal-mRNA complex, leading to a frameshift mutation and defective protein synthesis; leading to cell death.

Humans have ribosomes which are structurally different from those in bacteria, so the drug does not have this effect in human cells. At low concentrations, however, streptomycin only inhibits growth of the bacteria by inducing prokaryotic ribosomes to misread mRNA. Streptomycin is an antibiotic that inhibits both Gram-positive and Gram-negative bacteria, and is therefore a useful broad-spectrum antibiotic.



Streptomycin

Biosynthetic pathway:

Streptomycin is directly derived from glucose. Though the enzymes involved in the synthesis of N-methyl glucosamine are not yet known, it is expected that about 28 enzymes take part in the conversion of glucose into streptomycin.

Fermentation Process:

The Inoculum Production:

Spores of S. griseus maintained as soil stocks or lyophilized in a carrier such as sterile skimmed milk, is employed as stock culture. The spores from these stock cultures are then transferred to a sporulation medium to provide enough sporulated growth to initiate liquid culture build-up of mycelial inoculum in flasks or inoculum tanks. After sufficient mycelial growth, it is fed to production fermenter.

Preparation of the Medium:

A production medium contains carbon source and nitrogen source. Glucose is one of the best carbon sources which helps in the greater yield of streptomycin, because it provides basic carbon skeleton for the streptomycin production. Apart from glucose, fructose, maltose, lactose, galactose, mannitol, xylose and starch can also be used as carbon source. Polysaccharides and oligosaccharides generally give low yields.

Peptones, soya extracts, meat extract, the residue from alcohol distillation, ammonium salts, nitrates and glycine may be used as nitrogen source. Magnesium, calcium, potassium, boron and molybdenum may be used as mineral source along with sulphates, phosphates and chlorides.

Phenylacetic acid, L-naphthalene acetic acid may be added as growth stimulating compounds. It is better to add proline into the medium which helps in high streptomycin production. Fats, oils and fatty acids may also be used along with glucose. If necessary, antioxidants such as sodium sulphate or starch or agar may also be added into the medium. There is no need of precursor in the production of streptomycin.

Fermentation:

Sterilized liquid medium with all the above substances is fed to the production fermenter. Appropriate volume of inoculum (4-5%) is introduced into it. The optimum fermentation temperature is in the range of 25 to 30°C and the optimum pH range is between 7.0 and 8.0. High rate of streptomycin production, however, occurs in the pH range of 7.6 to 8.0.

The process of fermentation is highly aerobic and lasts approximately for 5 to 7 days and passes through 3 phases:

(a) The First Phase:

It takes about 24 hours to 48 hours. Rapid growth and formation of abundant mycelium occurs during this phase. The pH rises to 8.0 due to release of ammonia into medium, due to proteolytic activity of S. griseus. Glucose is utilized slowly and little production of streptomycin is witnessed.

(b) The Second Phase:

It lasts for 2 days. Streptomycin production takes place at a rapid rate without increase in the mycelial growth. The ammonia released in the first phase is utilized, which results in the decrease of pH to 7.6-8.0. Glucose and oxygen are required in large quantity during this phase.

(c) Third Phase:

Cells undergo lysis, releasing ammonia and increase in the pH, which falls again after a period of continuous streptomycin production. Requirement of oxygen decreases and the contents of the medium including sugar get exhausted. Finally, streptomycin production ceases. A yield of 1200 micrograms per milliliter of streptomycin is obtained.

Harvest and Recovery:

- After completion of fermentation the mycelium is separated from the broth by filtration.
- Streptomycin is recovered by several methods such as solvent extraction, solvent evaporation, crystallization, vacuum dry.

By product:

- Vitamin B_{12}

Immobilization

Definition: "Immobilization means that the biocatalysts/ Whole cell are limited in moving due to chemically or physically treatment"

Benefits of immobilizing an enzyme:

- Multiple or repetitive use of a single betel of enzyme
- The ability to stop the reaction rapidly by removing the enzyme from the solution.
- Enzyme are usually the stabilized by bounding.
- Product is not contaminated with enzyme (especially in food and pharmaceutical industry).
- Analytical purpose - long half-life, predictable decay rates elimination of reagent preparation

Limitation:

- cost of carriers and immobilization
- changes in properties (selectivity)
- mass transfer limitations
    1) Problems with cofactor and regeneration
    2) Problems with multi enzymes systems
- activity loss during immobilization

Methods of Immobilization:

Immobilization methods may be sub divided in to various groups depending upon the physical relationship of the catalyst to the polymer may the techniques used to immobilize enzyme or cells are:

1) Adsorption

2) Covalent bonding

3) Cross linking

4) Entrapment

5) Encapsulation

Several hundred methods of immobilization have been describe some are more generally applicable than other and many more are yet to be discovered.

Polymer matrix: almost any material imaginable may be employed from cellulose hydro gels to materials such as nylon, glass or even iron fillings.

1. Adsorption

Physical adsorption of an enzyme to a solid is probably the simplest way of preparing immobilize enzymes. The method relies on non-specific physical interaction of the matrix, brought about by mixing a con. Solution of enzyme with the solid. A range of non-specific or specific bonding force may be employed for example electrostatic hydrophobic interaction or affinity bonding to specific ligands attached to the polymer. One of the most widely used immobilization process is that of electrostatic bonding of the catalyst to ion exchange cellulose such as DEAE cellulose.

Advantages:

- No reagents and only a minimum of activation steps are required and as a adsorption less disruptive to the enzyme.
- Availability of ready prepared polymer material suitable for use for use in column reactor.
- Potential for regeneration of the immobilized catalyst
- Application to whole cells or organelles

Disadvantages:

- Leaching of enzyme or cells occurs relatively easily with change in pH, ionic, strength.
- Non-specific adsorption also allows adsorption of other substances this may alter the properties of the immobilized enzyme.

2. Covalent bonding:

In this technique there is a formation of covalent bond between the enzyme and support matrix.

In principle there are two ways of covalent bonding an enzyme to a polymer. By activating the polymer with a reactive group. By the use of a bifunctional reagent to bridge between enzyme and polymer.

Activating the polymer:

The groups on the enzyme through which it is coupled are -OH,-NH2 and to a lesser extent sulfhydryl group. But this causes inactivation of enzyme because of conformational changes on enzyme's active site. Polymers used here are hydro gels like cellulose or polymers used here are hydro gels like cellulose or poly acryl amides on to which diazo, carbodiimide or azide groups are incorporated. such hydro gels can be activated with cyanogen bromide

Use of bifunctional reagent:

Two approaches are possible

1) Mixing together polymer (glutaraldehyde). enzyme and reagent.

2) Reaction of bifunctional group with the polymer to the desired degree of substation and then enzyme is added so, bifunctional reagent is effectively prevented from penetrating the enzymes active site. (Seen in box)

Here, second substitution of -OH group of ethanolamine allows.

1) Introduction of positive, negative or charged group to alter the ionic properties of polymer

2) Alteration in properties of final immobilized enzyme preparation.

Metal ion complexes like titanium are also used to link catalyst and polymer.

Advantages:

- Easy and cheap method
- Potential to regeneration of immobilized catalyst.
- Technique of catalyst is not a problem.
- The polymer can be construed to carry any sign or degree of charged groups.
- Almost any material can be used as polymer to immobilize any enzyme.
- The ability to fix enzyme to soluble polymer.

Disadvantages:

- Frequent inactivation of enzyme.
- The use of toxic reagent
- Often complicated preparative routines.

3. Cross linking:

Immobilization of enzyme has been achieved by intermolecular cross linking of protein either to other protein molecules or to itself by reaction with a bifunctional group reagent.

The bifunctional reagent most often used is glutaraldehyde. Generally cross linking is best used in conjugation in other method.

Advantages:

- The technique is cheap and simple.
- It yields very little bulk of immobilized enzyme with very high intrinsic activity.
- It is widely used in commercial preparation such as glucose isomerase.

4. Entrapment:

The entrapment method of immobilization is based on the localization of an enzyme within the lattices of a polymer matrix or membrane. The biocatalyst is dissolved in a solution of the polymers precursors and polymerization initiated.

Two types of polymer can be used

1) Polyacryalamide type gel

2) Naturally derived gel materials such as agar, cellulose acetate, gelatin, carrageenan or alginal.

Advantages:

- Simple method
- Mild condition used
- Useful for immobilizing cells

Disadvantages:

- It needs to keep the average pore size of the gel as high as possible to prevent excessive diffusion limitation and the variability of pore size in such gels.
- There may be great linkage of biocatalyst from the gel, particularly with low Molecular weight enzymes.
- Nonviable cells are usually retained well, but viable the viable dividing cells may burst from the gel material.
- None of the entrapment method used is perfect.
- Polyacrylamide gel: The monomers from which is it formed and the free radicals generated during polymerization are toxic.
- Agar and carraseenan: Have large pore size (>10micro) causing escape of the cell. They are depolymerized by mild heat.
- Calcium alginate: Used for viable cell immobilization they are disrupted by calcium chelating agent such as citrate, or phosphate which might be include in the reaction medium. One novel variation of this technique involves the preparation of fibers immobilized enzyme. e.g. fiber of beta galactocidase entrapped in cellulose triacetate for use in the hydrolysis of lactose in milk and more recently a technique of spinning fibers of calcium alginate entrapped cells has been patented.

5. Encapsulation:

Micro encapsules are usually spherical particles where a liquid or suspension is enclosed by a dense but semi permeable membrane. Encapsulation of biocatalyst by enclosing a droplet of solution of biocatalyst in a semi permeable membrane capsule has to date largely been restricted to medicated to medical application. The technique is simple and cheap, but to be effective the biocatalyst must be stable in solution.

Here, little diffusion limitations are encounter and capsules of defined size can be formed easily Material used to form the capsule may be permanent such as nylon, polylaetic acid or phospholipid liposome. Although the catalyst is effectively retained in a capsule, such preparations are mechanically unstable.

Advantages:

- This technique provides large surface area to volume ratio.
- It is easy to perform.
- Allows easy replacement of the enzyme containing substrate streams which might block reactors based on other forms of immobilization.

Disadvantages

- Enzyme is no way protected
- No potential for enzyme stabilization
- Adsorption of enzyme onto the walls of fibers cause denaturation.
- It is limited by the molecular weight of the substrate (i.e., if high MW, the substrates could not pass the capsule to reach to Enzyme)

Choice of immobilization method:

When immobilization an enzyme on a surface. It is most important to choose a method of attachment aimed at reactive group outside the active catalytic use to binding site of that enzyme.

Considerable knowledge of active site of particular enzyme will enable methods to be chosen that would avoid reaction with the essential groups therein

Alternatively active sites can be protected during attachment as long as the protective groups can be removed without loss of enzyme activity.