# Chapter 8
# Industrial Production of Antibiotics and Organic Acids

## Production of Antibiotics

Antibiotics are chemicals that kill or inhibit the growth of bacteria and are used to treat bacterial infections. They are produced in nature by soil bacteria and fungi. Antibiotics are the most widely used chemotherapeutic agents. Most commonly used types of antibiotic are Aminoglycosides, Penicillins, Fluoroquinolones, Cephalosporins, Macrolides and Tetracyclines. Five basic mechanisms of antibiotic action against bacterial cells are inhibition of nucleic acid synthesis, inhibition of protein synthesis, inhibition of cell wall synthesis, alteration of cell membranes and antimetabolite activity.

## Penicillin

Penicillins are a group of antibacterial antibiotics produced by the fungus *Penicillium notatum* and *Penicillium chrysogenum*. The penicillin producing bacteria were firs identified by Alexander Fleming in 1926. Penicillin was the first important commercial product produced by an aerobic, submerged fermentation. Like all antibiotics, penicillin is a secondary metabolite, so is only produced in the stationary phase. Penicillin are effective against actively growing gram-positive bacteria. Some penicillin like amoxicillin are also effective against gram-negative bacteria except *Pseudomonas aerugionsa.*

## Industrial Production Techniques

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


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## Penicillin Production Process

Penicillin is commercially produced in the industry by culturing the fungus *Penicillium chrysogenum* or *Penicillium notatum*. Penicillin production is previously achieved by surface process i.e, solid state fermentation and surface liquid fermentation. Nowadays commercial production is carried out by fed batch process.

## Inoculum Preparation

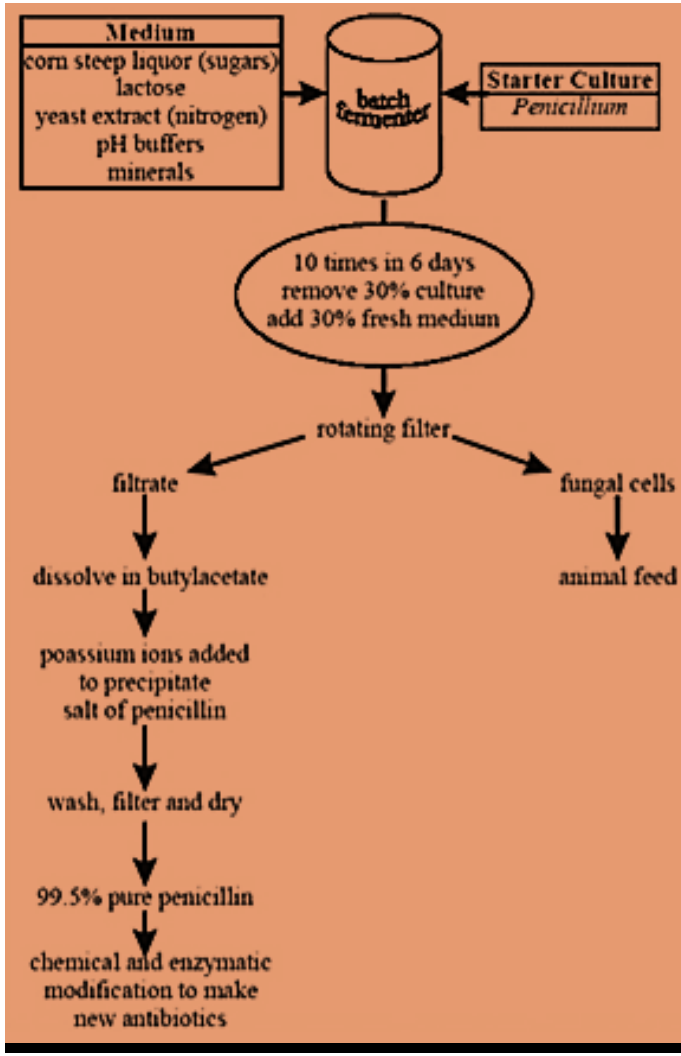
For inoculum preparation, spore from heavily sporulated working stocks are suspended in water or non-toxic wetting agents (Sodium sulfonate 1:10000). These spores are then added to flask containing wheat bran and nutrient solution for heavy sporulation. Incubated at $24^0$C for 5-7 days. Spores are then transferred to seed tank and incubated for 24-48 hrs at $24^0$C with aeration and agitation for sufficient mycelial growth. These mycelia can be used for production fermenter.

## Fermentation

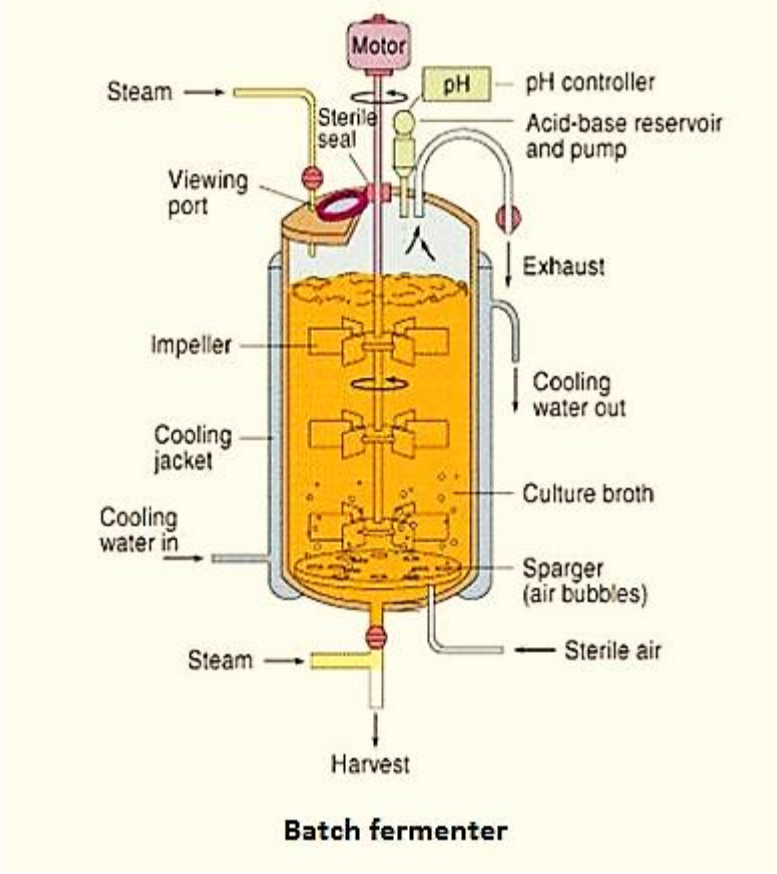
The method used is fed-batch or batch fermentation. Jackson in 1958 prepared a media for penicillin production. The major constituent of typical medium includes,

| | | |
|---|---|---|
| 1) Fermentable carbohydrate | - | Corn steep liquor (3.5%), lactose (3.5%), glucose (1%). |
| 2) Potassium dihydrogen phosphate | - | 0.4% |
| 3) Organic nitrogen source | | |
| 4) Phenyl acetic acid precursor | | |
| 5) Edible oil | - | 0.25% |
| 6) Calcium carbonate (act as buffer) | - | 1% |

Stirred tank or air lift tank fermenter is used. pH is adjusted to 5.5-6.0 which increased up to 7-7.5 (optimum) due to liberation of ammonia gas and consumption of lactic acid. If pH is 8 or more, $CaCO_3$ or $MgCO_3$ or phosphate buffer is added. Temperature employed for the penicillin production is $28^0$C. Supply of oxygen in a bioreactor is the limiting factor in penicillin biosynthesis. Aeration speed is between 3.0-1.5$m^3$. Production of penicillin depends up on biomass production, therefore it is desirable to have a high biomass concentration in the vessel. It is achieved by increasing the agitation rate and power.




**Industrial production of penicillin**




## Recovery and Purification

Harvested culture broth includes penicillin G along with a variety of other metabolites. Vacuum filter is used for separation of mycelium from the broth. Conversion of penicillin to the anionic form occurs at low pH (2.0-2.5). The lowering of pH is done by adding phosphoric acid or sulphuric acid. For the removal of pigment and other impurities from solvent containing penicillin it is treated with active charcoal. The product is back extracted into water from solvent by adding sodium hydroxide. The product of penicillin is then crystallized into sodium or potassium penicillin.

## Streptomycin

Streptomycin is an antibiotic produced by the bacteria, *Streptomyces griseus*, is active against gram-negative bacteria and against tuberculosis bacterium,

*Mycobacterium tuberculosis*. However, it is proved to be useful in the treatment of infections caused by gram-positive specially resistant to penicillin. It is also useful in the control of plant disease caused by bacteria as it acts systemically in plants.

## Industrial production

Industrially streptomycin is produced by submerged culture method.

## 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 the 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 mycelilal growth, it is fed to production fermenter.

## Preparation of Medium

A production medium contains carbon source and nitrogen source. Glucose is one of the best carbon sources which helps in increased yield of streptomycin. Apart from glucose, fructose, maltose, lactose, galactose, mannitol, xylose, and starch can also are used as carbon source. Peptone, soya extracts, meat extract, residue from alcohol distillation, ammonium salts, nitrates and glycine may be used as nitrogen source. Phenylacetic acid, L-naphthalene acetic acid may be added as growth stimulating compounds. It is also 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.

## Fermentation

Sterilized liquid medium is fed to the fermenter. Inoculum of appropriate volume is introduced into the fermenter. The optimal fermentation temperature is in range of 25 to $30^0$C and optimum pH range is between 7.0 - 8.0. The fermentation is highly aerobic and lasts 5-7 days and passes through 3 phases.

## The first Phase

It takes about 24 hours to 48 hours. Rapid growth and formation of abundant mycelium occurs. Here the pH rises to 8.0 due to the release of ammonia into medium and due to the proteolytic activity of *S. griseus*. Glucose is utilized slowly and little production of streptomycin occurs.

## The second phase

It lasts for 2 days. Streptomycin production occurs at a rapid rate without increase in the mycelial growth. 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.

### The third phase

Cells here undergo lysis, releases ammonia and pH increases which falls again after a period of continuous streptomycin production. Oxygen requirement decreases and the contents of the medium including sugar get exhausted. Finally streptomycin production stops. The 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. Generally employed method is the fermentation broth is acidified, filtered and neutralized. It is then passed through a column containing a cation exchange resin to adsorb the streptomycin from the broth. The column is than washed with water and the antibiotic is eluted with hydrochloric acid or cyclohexanol or phosphoric acid. It is then concentrated at $60^0$C under vacuum.

The streptomycin is then dissolved in methanol and filtered and acetone is added to the filtrate to precipitate the antibiotic. The precipitate is again washed with acetone and vacuum dried. It is purified further by dissolving in methanol. The streptomycin in pure form is extracted as calcium chloride complex.

### By-product Vitamin B12

Vitamin B12 is produced as a by-product which will not affect adversely the yield of streptomycin.

## Acetic acid

Industrial production of Acetic acid

Acetic acid is systematically known as ethanoic acid.

- It is a colourless liquid organic compound
- It has a pungent smell
- It is the principle constituent of Vinegar
- The first Vinegar was Spoiled wine
- Glacial acetic acid is the pure form of acetic acid (99.98%)
- Vinegar is product of Acetic acid

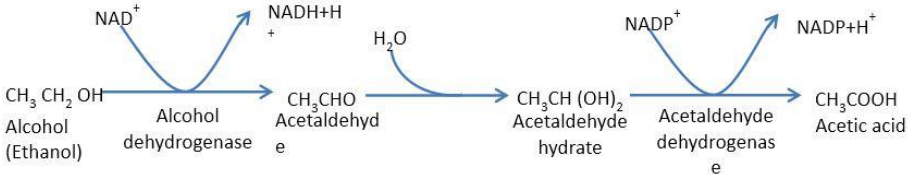
### Microorganisms used for the production of Acetic Acid

The commercial production of acetic acid is carried out by a special group of

acetic acid bacteria, which are divided into two genera *Gluconobacter* that oxidizes ethanol exclusively to acetic acid. *Acetobacter* that oxidizes ethanol first to acetic acid, and then to $CO_2$ and $H_2O$. Acetic acid bacteria are gram-negative bacteria. Eg: *A. aceti* , *A. peroxidans*, *A. pasteurianus*.

### Biosynthesis of acetic acid

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




**Biosynthesis of acetic acid from ethanol**

### Production process for Acetic acid

For every molecule of ethanol oxidized, one molecule of acetic acid is produced. Thus high yielding strains can produce 11-12% acetic acid from 12% alcohol. For optimal production, adequate supply of oxygen is very essential. Insufficient oxygen, coupled with high concentration of alcohol and acetic acid result in the death of microorganism. Surface fermentation or submerged fermentation process can be carried out to produce acetic acid.

### Recovery

The acetic acid produced is clarified by filtration and then subjected to decolorization by $K_4[Fe(CN)_6]$.

## Vinegar

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

## Surface process

The fermentation material is sprayed over the surface which trickles through the shavings that contain the acetic acid producing bacteria. The temperature is around $30^0$C on the upper part while it is around $35^0$C on the lower part. Vinegar is produced in about 3 days.

## Submerged process

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

## Raw materials

- Biomass feedstocks (beach wood, silicon tube, sieving, wood chips)
- Ethanol is used as substrate

## Microbes

Acetic acid bacteria (*Acetobacter aceti*) used as bioreactor. The production of Vinegar actually involves two fermentation processes – the first utilizing yeast to produce alcohol from sugar and the second utilizing acetic acid bacteria to oxidize ethanol to acetic acid through acetaldehyde. The microbial oxidation of ethanol to acetic acid is an aerobic fermentation that has high oxygen requirement. *Acetobacter* bacteria are employed for the industrial production of vinegar. *Acetobacter* bacteria can be divided into 2 groups – *Gluconobacte*r and *Acetobacter*. *Gluconobacter* oxidizes ethanol to acetic acid while *Acetobacter* oxidizes ethanol first to acetic acid and then to $CO_2$ and $H_2O$. Species of the *Acetobacter* used commercially are *Acetobacter aceti* and *A. pasteurianum*. Similarly, *Gluconobacter oxydans* and its subspecies are employed in the commercial production of vinegar. Two oxidation steps occur during the conversion of ethanol to acetic acid. In the first step ethanol is oxidized to acetaldehyde in the presence of NAD or NADP and in the second step acetaldehyde is changed to acetic acid under the catalytic action of enzyme alcohol dehydrogenase.

## Fermentation

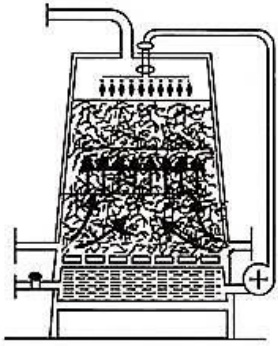
Commercially acetic acid is produced by two methods namely surface fermentation process and submerged fermentation process. Nutrient concentration that is used in submerged fermentation is generally five times greater than surface fermentation.

## Surface fermentation process

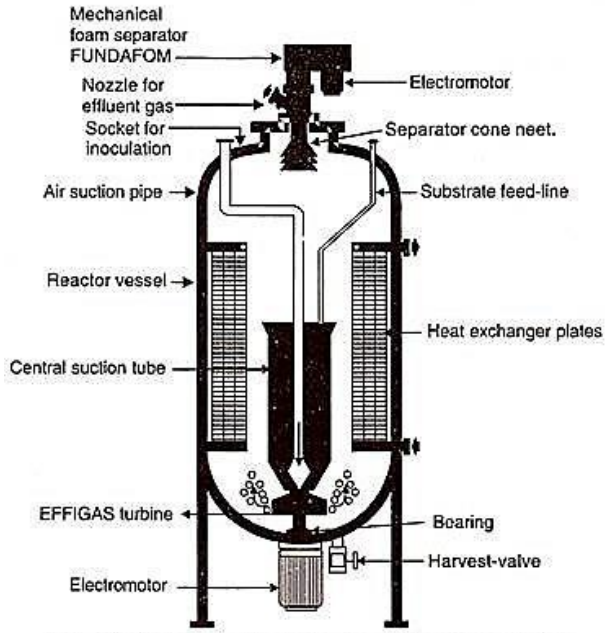
Tricking generator is generally needed in this process.

It is made up of wood and has a total volume up to $60m^3$ and its inner surface is lined with birch wood shavings. The starting material that is ethanol is passed into the generator from top which trickles through the birch wood shavings containing

bacteria into bottom basin where the partially converted solution is cooled and pumped back again to the top of the generator and passed again through it. This process is repeated again and again until 88-90% of alcohol is changed to acetic acid. The starting material should contain both acetic acid and ethanol for optimal growth of *Acetobacter*. Presently higher yielding strains are employed in vinegar fermentation which are able to yield 13-14% of acetic acid.



**Trickling generator for acetic acid production**




**Submerged fermenter for acetic acid production**

## Submerged fermentation process

Material with low alcohol concentration such as fruits, wines and special

mashes were first used in the initial stages of submerged fermentation process, which generally do not require aeration. But presently high yielding materials are employed which are capable of yielding 13% acetic acid. However the process with such high yielding material requires high aeration up to $50m^3$ oxygen. Fermenter constructed with stainless steel are employed and they are stirred from bottom. Aeration is provided with a suction rotor, with the incoming air coming down through a pipe from the top of the vessel. Heat exchanger is provided to control the temperature along with foam eliminators. The fermentation process is carried up to 35 hours at $40^0C$ temperature. The yield of acetic acid is about 98% in fully continuous process.

Recovery

The Vinegar produced in a submerged fermentation process is turbid due to the presence of bacteria. It is clarified by filtration. Plate filters and filter aids are generally used. After filtration $K_4$ [Fe (CN)6] is used to decolorize the final product, if required.

## Citric acid

### Production of citric acid

Citric acid can define as the most common weak organic acid found naturally in lemon fruits. The production of citric acid is an industrial process, which makes the use of raw materials like substrates, citric acid growth promoting microorganisms and enzymes etc for the commercial production of citric acid. Generally, their commercial production are achieved by employing the method of fermentation. The commercial production of citric acid yields significant importance because its use has been constantly increased the human consumption by 4% each year and it is having high demand in pharmaceuticals, food and other industries like cosmetic, toiletries etc. In the year 1826, the commercial production of citric acid was firstly achieved by the John and Edmund Sturage Company, U K. Citric acid production involves natural process and synthetic process. In natural process, citric acid is naturally produced from citrus plants like lemon, orange etc. In synthetic process citric acid is chemically synthesized by the enzymes and biological fermentation by microorganisms.

### Production process

- Biochemical method by fermentation
- Biological method by chemical reactions

### Biochemical method

Citric acid produces as a primary metabolite by the microorganisms. The citric acid produces at the time of tropophase cell growth as a result of defective citric acid or krebs cycle. In defective krebs cycle, a high amount of sugar is transported through EMP pathway that forms acetyl-CoA. The acetyl-CoA condenses with oxaloacetic acid to yield "citric acid" by the help of citrate synthase enzyme. Therefore

for the production of citric acid, the enzymes of the Krebs cycle must be deactivated like enzyme Aconitase/Isocitrate dehydrogenase which can further break down the citric acid.

## Biological method

Biological method involves the fermentation of citric acid by the use of microorganisms.

## Fermentation of citric acid

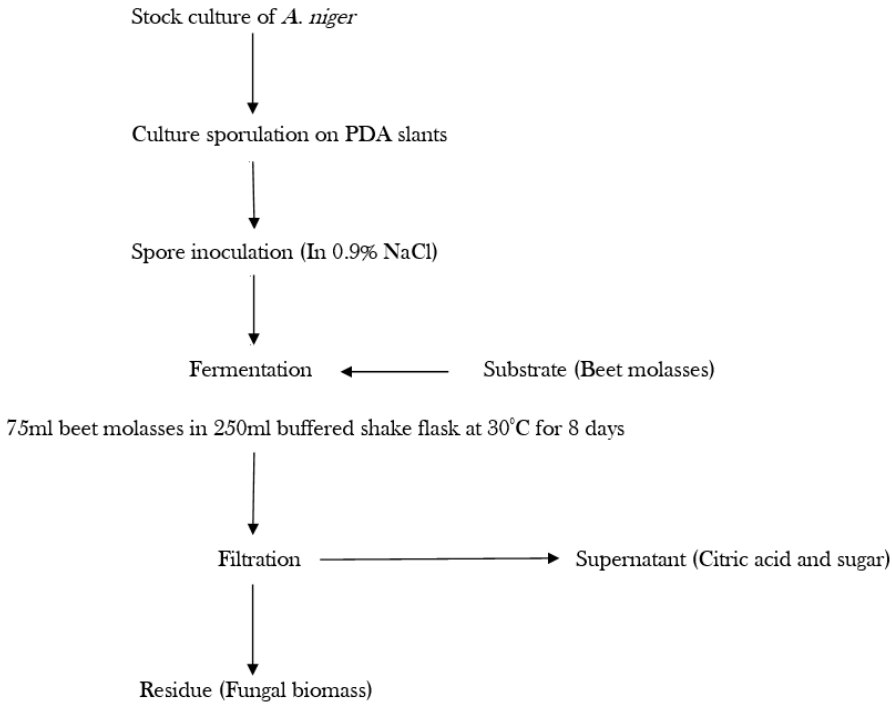
- Koji process
- Surface culture process
- Submerged process

## Koji Process

Koji process also known as **"Solid- State Fermentation"**. This process was first introduced in Japan. It is related to the use of agro-industrial residues for citric acid production. Raw materials such as apple, pomace, sugar cane, beet molasses etc can be used. *Aspergillus niger* utilized these raw materials. The pH and moisture content of the raw material is adjusted to 4-5 and 70% respectively. The raw materials are than cooled at 30-60$^0$C and inoculated with *A. niger*. After inoculation, the medium is transferred into large trays of 3-5 cm depth and incubated at 25-30$^0$C for 3-7 days. Finally extracted the citric acid from the fermentation tank. The starch content of the raw material is degraded into citric acid by amylase enzyme of *Aspergillus niger*. Pre-treatment of substrate is not required in Koji process because the trace elements do not affect the citric acid production.

## Submerged culture process

Submerged culture process also known as **"Submerged Culture Fermentation"**. By using this submerged fermentation method about 80% of citric acid production is carried out. Submerged fermentation makes the use of black *Aspergillus japonicus*. It is performed in a bioreactor made of stainless steel compiled with cooling system, impellers, proper aeration etc. Substrates like beet molasses, corn starch etc used as carbon source. For the nitrogen source, ammonia is used. Pre-treatment is required for the substrate used like the addition of nutrients, sterilization etc. Inoculated the culture medium with *A. japonicus* and maintained at 30$^0$C. Submerged fermentation is mostly carried out in a batch bioreactor in which 1500 kg of citric acid and 500 kg of biomass can be produced from the 2500 kg glucose and 860 kg of oxygen.




Citric acid production by biological pathway

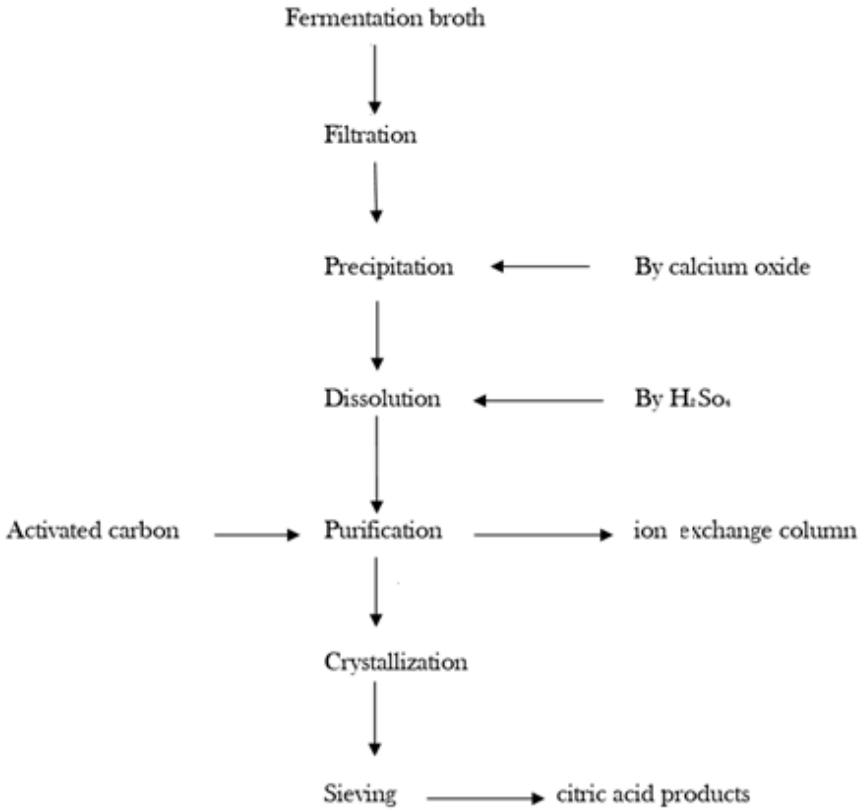
## Surface culture process

It is also known as **"Liquid Surface Fermentation"**. Surface culture fermentation was the first method introduced for citric acid production in 1919. In liquid surface fermentation, the culture medium of 5-6 pH is added to the aluminium shallow trays up to 5-20 cm depth. This process is carried out in fermentation chamber which provides uniform air circulation, maintains relative temperature and humidity. The spores of *A. niger* is firstly blown on to the surface of the culture medium for about 5-6 days and then dry air is passed. The pH of the medium is adjusted to 1.5-2.0. After 24 hrs, the spores started to germinate and the growth of white mycelium is observed on the surface of culture medium. After the utilization of sugar content by the mold, the remaining liquid is separated from the mycelial mat. In the surface culture process, a small amount of citric acid is produced as the primary metabolites by the *A. niger*.

## Recovery of citric acid

The product formed after fermentation is the fermented liquor looks hazy due

to the presence of antifoaming agents, mycelia etc. For the separation of these things, a slurry of Calcium hydroxide ie, $Ca(OH)_2$ is used and it form a precipitate of calcium citrate. The calcium citrate precipitate is filtered and washed. After filtration, it is treated with $H_2So_4$ for the precipitation of calcium as $CaSo_4$. Calcium sulphate is then treated with the activated carbon by which it gets demineralized after passing it consecutively from the ion exchange bed. The solution obtained is subjected to circulating crystallizers. Crystals are formed as a result of crystallization are then removed by centrifugation. The remaining solvent is then dried, sieved and then packed.




**Recovery of Citric acid**

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