INDUSTRIAL MICROBIOLOGY

UNIT:IV

Typical fermentation processes

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Content

- Citric acid
- L lysine
- Protease

Citric Acid Production

- **INTRODUCTION**
- Citric acid is the most important commercial product which is found in almost all plant and animal tissues.
- The molecular formula of citric acid is C6H8O7, 2-hydroxy-1,2,3-propane tricarboxylic acid.
- It is widely used organic acid in the field of food (60%) and pharmaceuticals (10%).
- In 1784 W.SCHEELE's first time isolated from lemon juice as calcium citrate, which treated with sulphuric acid gave citric acid in the liquid phase.

- **Microorganism used for Citric Acid Production**
- A large number of micro-organisms have been employed to produce citric acid. These include bacteria, fungi, and yeasts. But ***A.Niger,A.oryzae*** and *Saccharomycopsis* sp. are employed for commercial production because it has several advantages.

Citric Acid Cycle

- The citric acid cycle is also called the tricarboxylic acid cycle (TCA).
- The steps include in the citric acid cycle are:
- Formation of Citrate
- Formation of Isocitrate via cis-Aconitate
- Oxidation of Isocitrate to a-Ketoglutarate and CO2
- Oxidation of a-Ketoglutarate to Succinyl-CoA and CO2
- Oxidation of Succinate to Fumarate
- Hydration of Fumarate to Malate
- Oxidation of Malate to Oxaloacetate

- **Accumulation of citric acid**
- **By mutation**:
- Giving rise such a mutant organism that can carry an incomplete citric acid cycle for its accumulation
- **By inhibiting enzymes:**
- By altering environmental conditions (pH, Temperature)
- Treat medium with ferrocyanide or ion exchange versions so that the enzymes involved in the TCA cycle inhibit except citrate synthase.

Strain selection:

Strain with positive characteristics selected.

Genetic stability, high yield, amount of sporulation,lacking ability to degrade the citric acid product. Sporulation , Shu Jonhnsons

Socks are maintained and stored in the form of dry spores.

Fermentation medium:

1.Nutrient deficiency : in the form of trace metal ions and phosphate s required. It varies with strain.

Medium should be slightly deficient in phosphate, iron ,manganese, zinc and copper.

Addition of methanol reduce toxicity.

Fermentation medium:

Beet molasses medium

Sugar :10-20%,

Ammonium nitrate, Magnesium sulfate, and KH2PO4 added to the medium.

Beet molasses contain great quantity of trace metal ions hence pre-treated with ferricyanide, cation exchange resins

The industrial citric acid production can be carried in three different ways:

- Surface fermentation
- Submerged fermentation
- Solid-state fermentation

SURFACE FERMENTATION PROCESS

- Molasses substrate(15-20% of sucrose, added nutrients) acidified with, phosphoric acid to a pH 6.0-6.5 and heated at T 110c for 15 to 45 min.
- Inoculation is performed by using spore suspension mixed with water or blowing spores sterile air and spread as an aerosol over the trays.
- The temperature is kept constant at 28-30 degrees during the fermentation by means of air current
- Within 24 hours after inoculation, the germinating spores form surface mat of mycelial growth .
- As a result of the uptake of ammonium ions from ammonium sulfate the pH of the substrate falls to 2.5

The fermentation process stop after 8-14 days. Recovery of mycelium to extract citric acid.

SOLID STATE FERMENTATION

- The solid substrate is soaked with water up to 65-70% of water content. After the removal of excess water, the mass undergoes a steaming process.
- Sterile starch paste is inoculated by spreading *Aspergillus niger* spores in the form of an aerosol or as a liquid conidia suspension on the substrate surface
- The pH of the substrate is about 5-5.5 and incubation period is 28-30 degree C.growth can be accelerated by adding Alpha-amylase. although the fungus can hydrolyze starch with its own alpha-amylase. During the citric acid production pH dropped to values below 2.
- The solid-state surface process takes 5 to 8 days at the end of which the entire is extracted with hot water. In other cases, mechanical passes are also used to obtain more citric acid from the cells.
- These all steps followed during the solid-state fermentation to produce citric acid

SUBMERGED FERMENTATION

- Beet molasses substrate (12-15%, reducing sugar content) nutritive salts, such as ammonium nitrate or potassium dihydrogen phosphate are added, pH of the substrate is maintained at 5.5 to 5.9.Stirred tank
- Inoculum prepation-Slant—suspension—
- 5-10% Pellets
- The development of the hyphae and the aggregation generally requires a period from 9 to 25 hours at room temperature
- Incubation period for Fermentation is 6-8 days. .
- Citric acid is fermentation is harvested.

Harvest and Recovery

- Filteration: mycelia separated

Calcium carbonate---calcium citrate—

- addition of sulfuric acid---release calcium as calcium sulfate.
- Uses: food –acidulent,
- cabonated beverages,
- Pharma
- Metal cleaning.

Production of L-Lysine:

Total world production of L-lysine is around 35,000 metric tons per year.

Industrially it is produced by two different fermentation methods. They are:

(a) Indirect fermentation: Meso DAP- *A.aerogenes* -L –LYSINE, Dual

(b) Direct fermentation.---L -LYSINE

(a) Indirect Fermentation: Dual

- It is also called as dual fermentation as two different microorganisms are employed in this fermentation process. Auxotrophic mutant of Escherichia coli is used in the first half of the fermentation and wild type or prototrophic *E. coli* or *Aerobacter aerogenes* is employed in the second half of the fermentation.
- Diaminopimelic acid produced in the first half of fermentation by auxotroph of *E. coli*, is converted into L-lysine by *A. Aerogenes* in the second half of the fermentation .A. Aerogenes should also be deficient of lysine decarboxylase so that further decarboxylation of lysine to cadaverine is prevented and accumulation of lysine is facilitated.

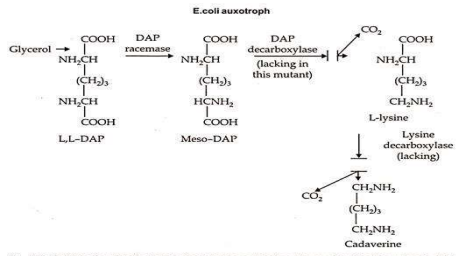

Fig. 5.2: Position of metabolic block in the L-lysine metabolic pathway of an *E.coli* auxotroph which accumulates diaminopimelic acid (DAPA) during growth on glycerol

- **(b) Direct Fermentation:**
- L-lysine can also be fermentatively produced from any of the substrates directly and the process is called as direct fermentation. Direct fermentation processes are presently employed throughout the world for the production of L-lysine.
- Direct production of l-lysine from carbohydrate was developed first with a homoserine or threonine plus *methionine* auxotroph of *Corynebacterium glutamicum.* Production of lysine by this bacterium is regulated by the mechanism as shown in Fig.

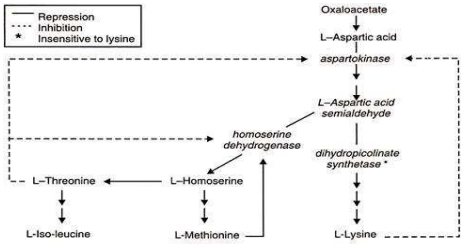

Fig. 5.4: Control of L-lysine production in *Corynebacterium glutamicum*

- Double auxotrophs, which require at least one of the amino acids, threonine or isoleucine or methionine in addition the homoserine, for growth have been found highly stabilized, showing little tendency to revert the homoserine independence.
- It is possible not only to prevent reversion of the culture to a wild type, but also to produce lysine in higher yields since many of the microorganisms are double mutants in the homoserine pathway.

Fermentation Process of L-Lysine:
This process consists of four stages.
They are:
(i) Preparation of inoculum,
(ii) Preparation of medium,
(iii) Fermentation process,
(iv) Harvest and recovery,

- **(i) Preparation of Inoculum:**
- Suitable and high yielding mutant strain of *C. glutamicum* usually (strain 901) is used from the stock culture for the production of inoculum. Seed cultures are raised twice, in which two different media are used.

Glucose	2%
Peptone	1%
Meat extract	0.5%
NaCl	0.25%

(ii) Preparation of Medium:

The medium with the following composition is used as fermentation medium. Reducing sugar (expressed as inverted cane molasses), 20%, Soyabean meal hydrolysate (as weight of meal before hydrolysis with $6NH_2SO_4$ 1.8% and neutralization with ammonia water) are dissolved in tap water and sterilized.

- **(iii) Fermentation Process: 5% Inoculum==5to 10%**
- The fermentation is carried out at 28°C and is allowed upto 72 hours. The amount of growth factors, homoserine or threonine and methionine should be appropriate for the production of L-lysine and suboptimal quantity to support the optimal growth. The biotin concentration in the medium should be greater than 30 mg per liter.

Dual:

- **(i) Inoculum Production:**
- Pure inoculum of both mutant strain *E. coli* and *A. aerogenes* is produced from the suitable and high yielding stock culture. These microorganisms should lack the ability to produce diaminopimelic acid (DAPA) decarboxylase and lysine decarboxylase enzymes respectively, so that the DAPA and L-lysine produced will not be further metabolized by respective organisms.

- Both the organisms should also possess strong, L- diaminopimelic acid racemase activity to convert all residual L, L-diaminopimelic acid to meso-diaminopimelic acid.
- The composition of the medium which is employed for inoculum production is similar to fermentation medium. The cells of the organisms are separated from growth medium by centrifugation or sedimentation.
- **(ii) Preparation of Medium:**
- Both the inoculum and fermentation media contain glycerol, corn-steep liquor as carbon sources and ammonium hydrogen phosphate, as nitrogen source. In addition, calcium carbonate is also used in the production medium. The levels of all of the nutrients are kept lower in the inoculum medium. Apart from supplying carbon source, the corn steep liquor also provides L-lysine required for the initial growth of auxotroph of *E. coli.* The pH of the medium is maintained at neutral, to slightly alkaline level (pH 7.5 to 8.0)

(iii) Fermentation Process:

- The fermentation is carried out for 3 days at 28 to 30°C temperature.
- The concentration of L-lysine provided to *E. coli* is very important, because providing low quantities, less than optimum, results in the back mutation and more quantities results in the feedback control of lysine biosynthesis both of which badly affect the yield

Part I :

Through a sequence of enzymatic steps during first stage of fermentation glycerol is converted into L, L-diaminopimelic acid, which is partially converted into D, L- isomer and mesodiaminopimelic acid by the action of diaminopimelic acid racemase enzyme.

Thus, mesodiaminopimelic acid accumulate in the fermentation broth because auxotrophic *E. coli* lacks diaminopimelic acid decarboxylase enzyme.

Hence, it cannot be converted into L-lysine. The broth contains approximately 40% L, L-isomer and 60% mesoisomer of diaminopimelic acid.

PART II:

In the second half of the fermentation, 1-2 days old culture of *A. aerogenes* is added to the fermentation broth formed at the end of first fermentation process.

The microorganism is allowed to grow for one day at 24°C. After sufficient growth occurs toluene is added to the fermentation broth which causes lysis of cells of *A. aerogenes*, due to which the enzyme diaminopimelic acid decarboxylase is liberated into the fermentation broth.

The meso-diaminopimelic acid is completely converted into L-lysine by the action of diaminopimelic acid decarboxylase.

(iv) Harvest and Recovery:

After sufficient quantities of L-lysine is formed, lysed bacterial cells are removed from the fermentation broth by centrifugation.

Ion exchange chromatography

Extraction with organic solvents

Uses of L-Lysine:

L-lysine is useful in many fields:

1. L-lysine is an essential amino acid required for the human nutrition.
2. It is used as supplementary for cereal proteins.
3. Protein quality of certain foods like wheat (based foods) is improved by addition of L-lysine which results in the improved growth and tissue synthesis.
4. It is used as a nutraceutical.

Microbial Protease enzyme:

- Proteases are the second most important industrial enzymes after amylase.
- About 500 tons of the enzymes are produced per year.
- This can be produced commercially from bacteria and fungi.
- The proteases on the market include-alkaline, neutral and acidic proteases.

i. Alkaline protease:

- Many bacteria and fungi excrete alkaline protease and the most important producer are *Bacillus* strains like *Bacillus licheniformis, Bacillus amylotiquefaciens, B. megaterium, B. purilis* and *Streptomyces* strains like *Streptomyces fradiae, S. griseus, S. rectus* and fungi like *Aspergillus niger, A. sojae, A. oryzae, A. flavus*
- Alkaline proteases are commonly used in detergents.
- Mainly proteases from *Bacillus licheniformis.*
- Alkaline proteases have some features which makes its applications in industrial scales like stability at high temperature, stability in alkaline range (pH 9-11), stability in association with chelating agents.

ii. Neutral protease:

- Neutral proteases are produced by bacteria and fungi. E.g. *Bacillus subtilis, B. coreus, B. megaterium, Pseudomonas aeruginosa, Streptomyces griseus, Aspergillus oryzae, Aspergillus sojae.*
- Neutral proteases are relatively unstable and calcium, sodium and chloride must be added for maximal stability.
- Its pH range for activity is narrow and sensitive to increased temperature.
- These are quickly inactivated by alkaline proteases.
- Because of these limitations, they have restricted industrial applications but can be used in leather industries and in food industries (For manufacture of breads and rolls).

iii. Acidic protease:

- Acidic proteases are found in animal cells, yeast and molds but never in bacteria.
- These microbial renin-like enzymes are derived from *Mucor michei, Mucor pusillus, Mucor racemaeus, Mucor bacilliformis.*
- Pepsin like acid proteases are derived from *Aspergillus species and Rhizopus spp.*
- Renin like proteases are commonly used in cheese production in optimum pH 2-4.

Applications of protease enzyme:

- Used in cheese production.
- Bating of hides in the leather industries.Textile industries ,silk ind
- Used in medicine (similar to mammalian pepsin)
- Used in digestion of soya-protein for soy-sauce production.
- Break down wheat gluten in baking industries.
- Laundry, removal food spots.

Production process of protease enzyme:

step I: Isolation of proteolytic microbes:

- Proteolytic microbes can be isolated by observing hydrolysis in **casein agar.**
- Among the various proteases, *bacterial proteases* are the most significant as compared with animal and fungal proteases.
- Among bacteria, *Bacillus spp* are specific producer of extracellular proteases.
- For industrial utilization, the genes for formation of several proteases have been cloned- protein engineering has been used to develop, modify *Bacillus subtilapeptideases* with altered amino-acid sequences.

Step II: Media formulation:

- Media rich in nitrogen sources such as soyabean milk, casein, gelatin and carbohydrate sources such as starch, or lactose are generally used for protease production.
- **step III: Fermentation:**
- The nature of fermentation, solid or submerged influences the growth of moss as well as enzyme production.
- For the production of alkaline proteases by using *B. subtilopeptidase* cultured are stored in lyophilized state or under liquid nitrogen (for sterility). Inoculum_5%
- Initial growth is carried out in shaken flask and small fermenter at 30-37ºC with aeration ,3 to 5 days. Harvested. centrifuged
- **Purification:**
- **Precipitation :Solvent extraction ,chromatography, dialysis`**
- Molecular exclusion

Amylase: bacteria,**fungi**,

Asp.oryzae
SSF:malting , Starch—glucose—Yeast
Wheat +gram=KOJI-*Asp.oryzae—Sporulation*
Mix in water- Solvent :acetone-ppt- enzyme
Dry: powder: enzyme=
Salt
Submerged: *B.subtilis*
Uses: sizing agent,textile ,

- Types: fungal and
- Bacterial :55

- https://www.google.com/url?sa=i&url=https%3A%2F%2Fwww.slideshare.net%2FSKV13%2Fcommercial-production-of-lactic-acid-75536743&psig=AOvVaw33kmAZZRwwgsIHYfKXbB1F&ust=1622349523659000&source=images&cd=vfe&ved=0CAIQjRxqFwoTCLi2gp2J7vACFQAAAAAdAAAAABAO