- Large Scale production of 1° and 2° Metabolites.

- **Primary Metabolites**

→ Metabolite / Molecules that is directly involved in normal growth, development and reproduction.
→ Metabolites are the intermediate products produced during metabolism.
→ Metabolites are produced by plants, humans and microbes.
→ 1° metabolite maintain physiological Functions of the body
→ Amino acids, vitamins, organic acids are examples of 1° metabolite.

① Vitamins.

→ Vitamin is a organic molecules.
→ Essential micronutrient that organism needs in small quantities For the proper Functioning of it's metabolism
→ Essential nutrients cannot be synthesised in the organism
→ Must be provided From outside.
→ Many micro-organisms synthesise vitamins.
→ Commercial production of vitamins is carried out by most of the microorganisms.

1. Vitamin $B_{12}$ (Cyanocobalamin).

→ Also know as ——.

→ Vitamin $B_{12}$ is a water-soluble vitamin with complex structure.

→ belongs to Cobalamin Family. composed of Corrinoid ring

→ Empirical Formula is $C_{63}H_{90}N_{14}O_{14}P$.

→ Vitamin $B_{12}$ Chemical Synthesis is not practical since it involves 20 complicated steps.

→ Fermentation is the only choise.

→ Vitamin $B_{12}$ synthesised by prokaryotes and inhibit development of pernicious anemia.

→ Microbial de-novo biosynthesis of vitamin $B_{12}$ occurs through two alternative routes. ① aerobic ② Anaerobic

→ Vitamin $B_{12}$ is manufactured by SmF

→ Aeration and agitation are important parameters for this process.

→ Fermentation process gets completed in 3 to 5 days.

**Micro-organisms Used**

→ Most commonly used micro-organisms are

① Propionibacterium Freudenreichii

② Pseudomonas denitrificans

③ Bacillus megaterium.

④ Streptomyces olivaceus.

Streptomyces griseus.

(Actinomycetes)

## Cobalamine Biosynthetic Pathway

Succinyl co-A → δ-amino levullinic acid

↓

Prophobilinogen.

↓

Uroporphyrinogen-III

↓

cobyrinic acid

↓

Cobinamide.

↓

5'- Deoxyadenosylcobalmine

↓

Ddenosyl cobalamine phosphate

↓

Adenosine GDP cobinamide

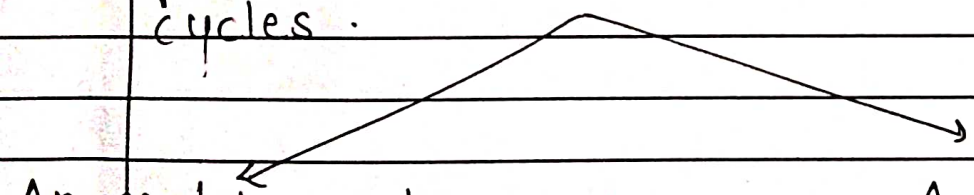
5, 6 dimethyl bezidamide. ↓

Vitamin $B_{12}$

- Vitamin $B_{12}$ production by Propionibacteria.

→ **Inoculum used**
i) propionibacterium Freudenrichii ATCC 6207
ii) Propionibacterium Shermannii ATCC 13673

→ **Medium**
i) Glucose
ii) Corn Steep liquor. → Lactic acid, penthathonic acid
iii) Cobalt. (5 µg/ml)
iv) Betain. (con. 0.5) → methyl group provided.
v) 5,6 dimethylbenzimidazole
→ PH - 7.5.
→ Temperature - 30°c.
→ Submerged Fermentation type is used.
→ process of Fermentation is divideded in two cycles.



Anaerobic cycle
→ 72 hrours
→ Formation of cobinamide.

Aerobic cycle.
→ 50 hours.

→ It is neccessory to add 0.1% 5,6 dimethy benzimidazole → links to cobinamide → to form cobalamine.
→ 1st phase anaerobic
→ 2nd is aerobic → with continous addition of 5,6 dimethyl benzidimazole.

- Production of $VB_{12}$ by using Streptomyces olivaceus NRRL B-1125.
- In the inoculum preparation, pure culture of streptomyces olivaceusis grown on Bennet's agar or SCDA.

→ **Inoculum used**

→ S. olivaceus NRRL B 1125.

→ **Medium Used**

→ Bennet's agar used.

| composition. | gm/lit |
|---|---|
| i) Beef extract | 1 |
| ii) Yeast extract | 1 |
| iii) N-Z Amine A | 2 |
| iv) Glucose | 10 |
| v) Agar | 15 |
| vi) D.W. | 15 |

→ **Fermentation process.**

→ culture is inoculated in Bennet's medium in Erlenmeyer Flask. and allow grocol by incubating on Shaker incubator.

→ Culture is obtain by transferring medium two three times.

→ In the production medium, 5% of this culture is added.

→ Production media used For vitamin $B_{12}$ production is consist of carbohydrate, protenaceous material, various salt and Source of Cobalt.

→ Cobalt needs to be added in the medium for the maximum yeild of Cobalamine.
→ Cynide is added to medium to help the conversion of Cobalmine to cynocaba imine.
→ Sterilization of medium is carried out batchwise or continously.
→

Batch wise
(Production tank is heated 121°c for one hour)

continously
(production tank is sterilized with charged tank).

→ Temperature of 27°c is optimum for fermentation process which gives satisfactory yeild of product.
→ Growth of Streptomycee is dependent upon aeration and agitation rate.
→ Aeration rate should be maintain optimum.
→ Higher aeration rate causes → Foaming.
→ Lower → Results in improper mixing.
→ Optimum rate is 0.5 volume/medium/mixture
→ Several antifoam agents are used to avoid Foaming
→ Contamination is controlled by proper sterilization.

## Recovery of Vitamin $B_{12}$.

→ At the end of Fermentation process, cobalmine is associated with mycelium and also present in solution.

→ Heating the mixture to boiling liberates the cobalmine From the mycelium.

→ 1st process in recovery is Filtration to remove mycelium.

→ Filtrate is treated with cynide For conversion of cobalmine to cynocobalamine.

→ Then Filtrate is passed through absorbing g-agent

→ Different absorbents like activated charcoal, ion exchange resins, bentonite

→ Finally elution of cyanocobdlimine For the absorbent is done with different solvents ranging From organic bases to hydrochloric acids

→ Chromatography is performed on alumina Followed by crystallization.

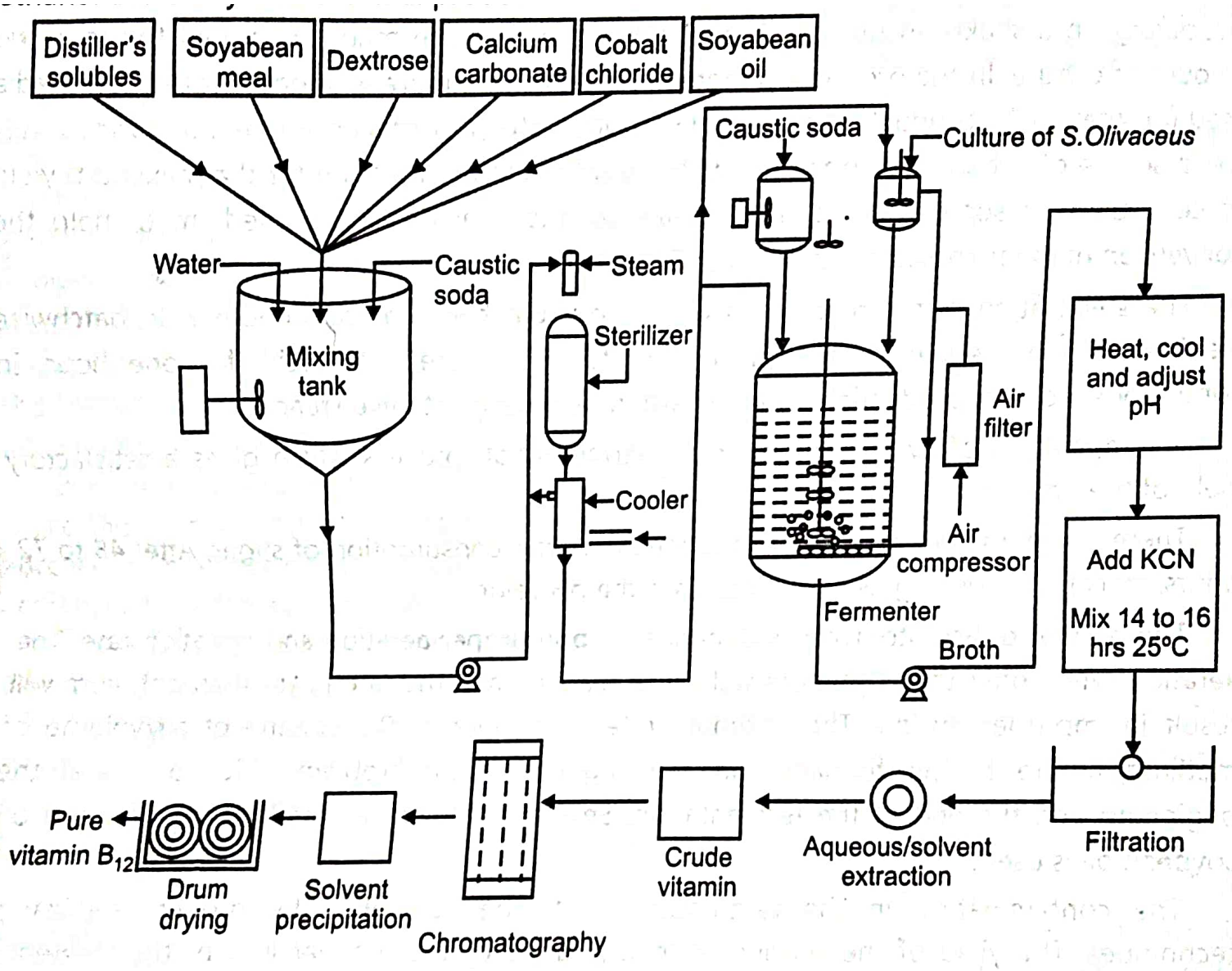



***Fig. 2.3: Flow Diagram for Production of Vitamin $B_{12}$***

# Riboflavin (Vitamin $B_2$)

→ Also known as Vitamin $B_2$, water soluble B-group vitamin.

→ Riboflavin is one of the essential micronutrients For human body.

→ average amount of riboflavin required For healthy person is 0.8 - 1.8 mg/day.

→ Riboflavin is an indispensible nutrient For normal cellular function.

→ After uptake it is intracellularly transformed into FMN and FAD.

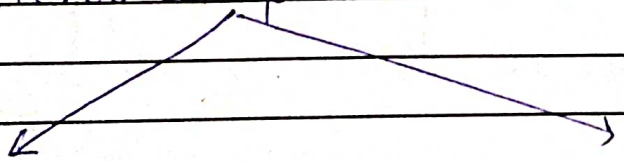
→ These biologically active riboflavin derivatives are involved in wide range of redox reactions that are crucial For human metabolism.

→

→ Current strategies For riboflavin production.

→ For production of riboFlavin, 3 are main strategies.

→ ① Total chemical synthesis.
② chemical semi-synthesis.
③ Microbial production.



① Traditional flavinogenic microbial Fermentation

② Fermentation by genetically engineered microbes.

1) Total chemical synthesis of riboflavin.

→ Total chemical synthesis employes D ribose or glucose as starting material and synthesises riboflavin via 6-9 chemical reaction steps.

2) chemical semi-synthesis of riboflavin.

→ riboflavin is combined strategy of microbial Fermentation and total chemical synthesis.

→ Microbial → D glucose applied as 1st

→ chemical → D-ribose use as 1st material

3) production of riboflavin by microbial Fermentation.

3 riboflavin producing microbes includes one bacterium (Clostridium acetobutylicum) two fungi (Ashbya gossypii, Eremothecium shybii) However → microbial fermentation processes not compete with chemical synthesis. due to long cycle and low yeild.

→ Later on with the reasearch and development of genetic engineering technology, genetically engineered bacteria here bech sucessfully Constructed.

→ this bacteria have capacity to transform D-glucose into riboflavin which can therefore significantly shorten the production cycle increase riboflavin yeild.

• Microbial Biosynthesis of Riboflavin.

→ Flavinogenic organisms such as fungi yeasts and eubacteria

1 molecule of GTP and 2 molecules of ribulose -5- phosphate

→ are required for the biosynthesis of one riboflavin molecule.

→ Current studies mainly focused on utilization of two yeast like Fungi Eremothecium ashbyii Ashbya gossypii

Three bacteria → Bacillus subtilis, E. coli and LAB.

→ For fungi → Riboflavin yeild may be improved by regulation of riboflavin biosynthesis → achived by chemical mutagenesis and genetic engineering.

→ Fungi uses Fatty acids → So supplemented with exogenous matter

→ Use of bacteria has several advantages

① Short fermentation process.

② Simple culture medium

③ Availability of genetic engineering technologies.

# Biosynthesis Riboflavin

GTP

↓ GTP cyclohydrlase II

2,5 Diamino -6- ribosylamino 4 3(H) pyrimidinone 5 phosphate (DARPP)

↓

5-amino-6 ribitylamino - pyrimidinone 5'-phosphate (ArPP).

↓

[In Fungi - DARPP $\xrightarrow{\text{reduced}}$ (results) DArPP

↓ Deaminated

ArPP]

[In eubacteria DARPP $\xrightarrow{\text{Deaminated}}$ ARPP

↓ Reduced

ArPP.]

The order of reduction and deamination reactions occurs oppositely in bacteria and fungi

In subsequent reactions ArPP → is dephosphorylated into ArP

ArPP —(non specific Phosphotase)→ ArP.

→ On other hand another initial substrate ribulose 5-phosphate can be transformed into DHBP (3,4 dihydroxy 2-butanone 4-phosphate)

DHBP Ribulose 5(P)

[DHBP converted through Skeletal
DHBP synthase.

→ Luminase synthase catalyses the condensation of

ArP + DHBP.

(Luminase synthase)

6,7 dimethyl ribityllymazine (DMRL)

transformed into riboflavin and ArPP.

(Riboflavin synthase)

via unusual dismutation catalysed by

## • Fermentation process.

There are 3 main aspects For riboflavin production.

① Preparation of culture medium.

② Selection of mutant culture and optimization of inoculum preparation.

③ Optimization of Fermentation conditions like PH, temperature, aeration level, fermenter design, etc.

→ Selection of suitable production medium is done as per the requirements of the producer strain.

[ Glucose, Corn Steep Liquor, Soyabean, Glycine ]

most suitable media For vitamin $B_2$ production Form Ashbya gossypii.

Fermentation time is - 96-120 hours.

Aeration agitation → imp parameters

Addition of antifoam agents.

## • Recovery of Riboflavin From fermented media

Upon completion of fermentation solids were dried to crude product.

→ When crystalline product was required, broth is heated for 1 hour at 121°c to solubalize riboflavin.

→ Insoluble matter was removed by centrifugation and riboflavin recovered.
→ ppt riboflavin then dissolved in water of polar solvents
→ Recrystallization from aqueous or polar solvents.

XX

## Amino Acids

### * (1) Glutamic Acid

→ chemically glutamic acid is 2 aminopentane dioic acid.
→ Molecular formula - $C_5H_9O_4N$
→ L-Glutamic acid was the 1st amino acid produced by micro-organisms (C. glutamicum)
→ Glutamic acid is used in production of monosodium glutamate (MSG) which is commonly known as 'Seasoning Salt'
→ MSG is condiment and flavour enhancing agent → commonly used in convenient food stuffs.
→

Strategies for synthesis of Glutamic acid
① By cleavage of pyrrolidone carboxylic acid found in Steffen's molasses.
② By hydrolysis of wheat ~~molasses~~ gluten, soyabean cake and other protein material
③ one stage fermentation process involved.

## * Selection of M.O.

~~Eg~~ C. glutamicum, C. Lilum, Brevibacterium divericu

→ Suitable Strain of C. glutamicum From stock culture is used For inoculum development Strain is inoculatied in Sterilized medium

→ Sufficient inoculum can be dexeloped and then added in Final production process.

## * Formulation of culture media.

→ Consist of carbon Sources, Nitrogen sources minerals, vitamins, Growth Factors, etc.

1] Carbon Sources - glucose, Sucrose, Fructose, maltose Sugar beet molasses, Sugar cane molasses Starch hydrolysate
generally cane molasses and Starch hydrolysate can be Used.

2] Nitrogen Sources.

Ammonium Sulphase, $NH_3Cl$, $NH_3PO_4$, aqueous ammonia, ammonia gas and Ured have been Used as nitrogen Source.
Souible / optimum amount of ammonia should be added as high amount inhibit growth of M.O as well as yeild of L-glutamic acid.
These salts helps in PH Control.

→ Phosphotase, vitamins and other neccessory supplements are usudly provided.

## • Growth Factors

→ Imp growth Factor is biotin (Its con. is influenced by carbon Sourse.

⇒ Composition of production medium.

| Ingredient | ~~con.~~ |
|---|---|
| Glucose | |
| Corn Steep liquor | |
| Enzymatic Casein hydrolysate | |
| $K_2HPO_4$ | |
| $MgSO_4 7H_2O$ | |
| Urea | |

# • Glutamic acid (continued.....)

• Effect of permeability on Glutamic acid production.

→ Glutamic acid is intracellular component

→ production and Extraction dependent on cell permeability of acid producing bacteria. Increased permeability can be done by

① Ensuring biotin deficiency in the medium.
② Treatment with Fatty acid derivatives.
③ Ensuring oleic acid deficiency in mutants requiring oleic acid.
④ Addition of penicillin during growth of glutamic acid bacteria (prevents cell wall formation)
⑤ Use of oleic acid and glycerol auxotro-ph.

→ Cell treated in one of the First three ways above have cell membranes in which Fatty acid ratio is abnormal ⟶ therefore permeability barrier is destroyed. Glutamic acid accumulates in the medium

→ Glutamic acid production ⟶ is greatest when biotin is sub optimal limiting.

→ When biotin is optimal ⟶ lactic acid is excreated.

→ Higher amount ⟶ glutamic acid production falls.

→ Isocitrate succinate → part of TCA cycle is needed for growth

* **Biosynthetic pathway of Glutamic acid**

→ **Corynebacterium glutamicum**

metabolises sugar via glycolysis.

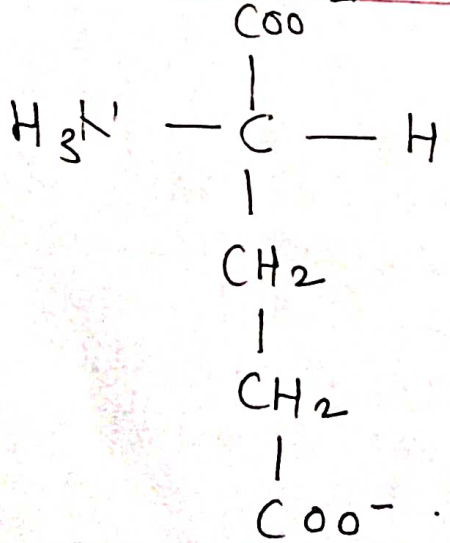
Formation of glutamic acid does not occur if inhibitor of glycolytic pathway are present.

→ Absence of α-ketoglutarate dehydrogenase

↓

lead to accumulation of Glutamate.

[ **Structure of Glutamate**.



]

Glucose

↓ Glycolysis.

Pyruvate.

↓

Acetyl co-A.

Oxaloacetate → citrate

↓

Iso-citrate.

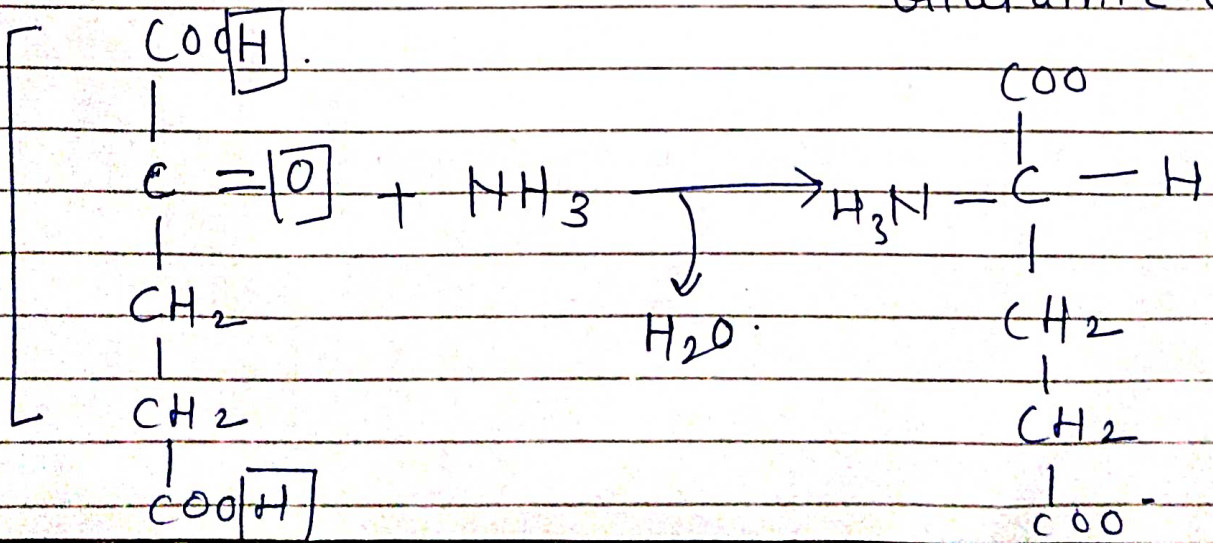
↓

α-ketoglutarate.

Succinyl CoA. ⊗

↓ Glutamate Dehydrgenase

Glutamic acid.



# Fermentation process.

→ The Fermentation process ~~starts with~~ includes microbial strain grown in fer-menters.

→ Fermentation medda is formulated using row materials.

→ Following Faetors affect glutamic acid Fermentation ① carbon source ② N source ③ Growth faetors ④ oxygen supply ⑤ pH of medium.

→ oxygen supply should be optimum.

→ low oxygen level → lactate and succinate accumulates.

→ High oxygen level → growth inhibition.

→ optimum pH For growth and production is 7.0-8.0. controlled by addition of ammonium salts.

→ Fermentation is carried out For 40-48 hrs at 30°c. temp. pH → 7-8.

## • Recovery process.

→ In down stream processing, bacterial cells are seperated
→ Broth is passed through anion exchange resin.
→ Glutamic acid anions get bound to resin and ammonia is released.
→ This $NH_3$ is recovered and used in fermentation.
→ E: treated with NaOH to form MSG.

## * Diagramatic Representation.

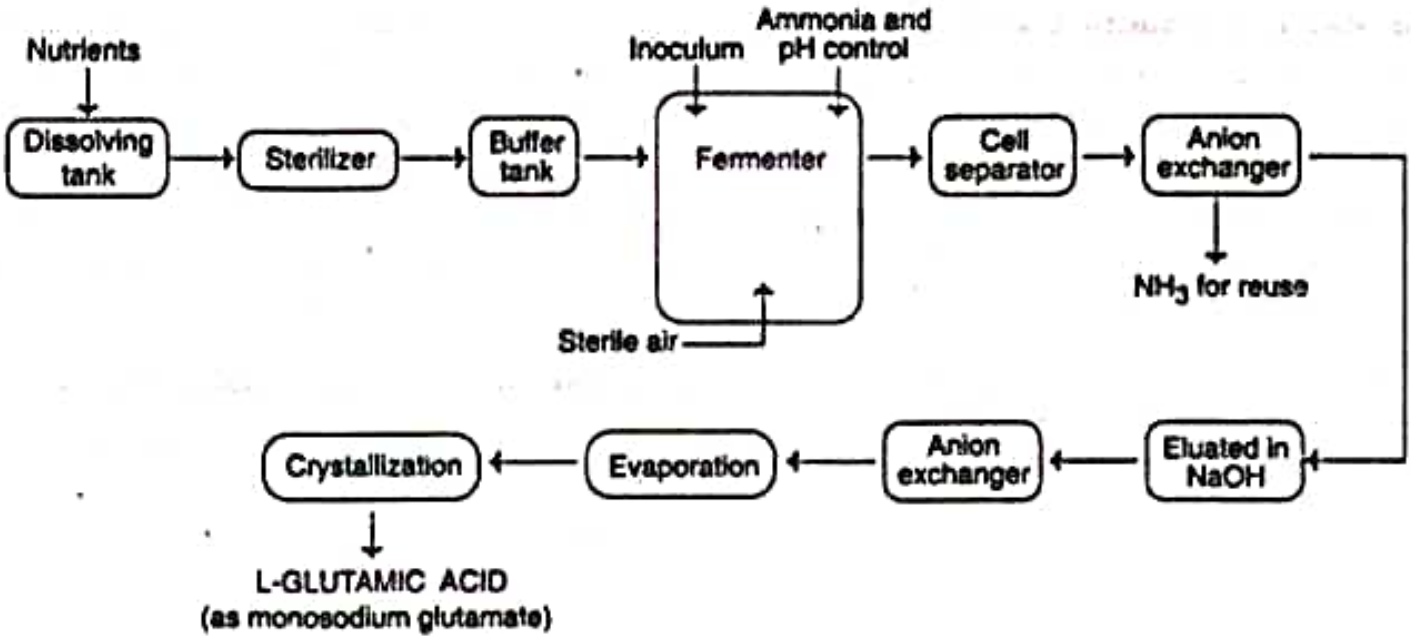



*Fig. 26.3 : Diagrammatic representation of glutamic acid production plant.*