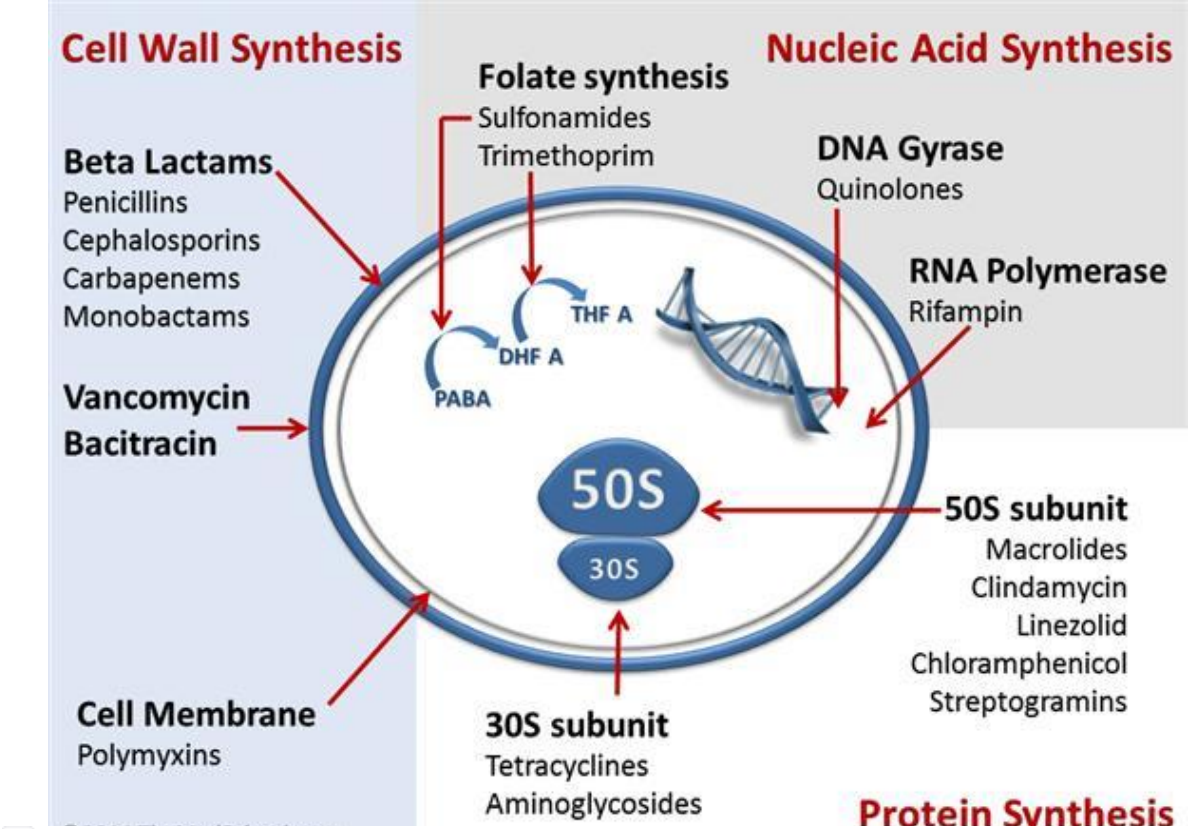
Secondary Metabolites

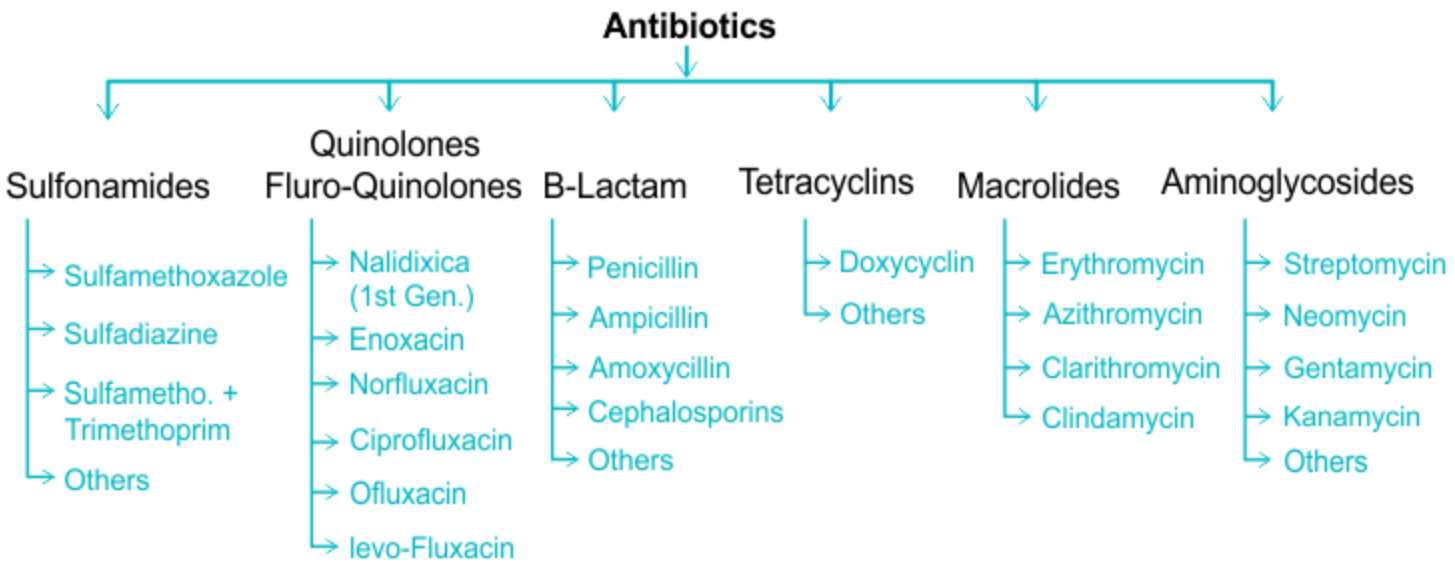
- Secondary metabolites are typically organic compounds produced through the modification of primary metabolite synthases.
- Secondary metabolites **do not play a role in growth, development, and reproduction** like primary metabolites do, and are typically **formed during the end or near the stationary phase of growth**.
- Many of the identified secondary metabolites have a role in ecological function, including defense mechanism(s), by serving as **antibiotic**s and by producing **pigments**.
- Secondary metabolism has no apparent function in the organism. The organism continues to exist if secondary metabolism is blocked by a suitable biochemical means
- Secondary metabolites are **produced in response to a restriction in nutrients**. They are therefore produced after the growth phase, at the end of the logarithmic phase of growth and in the stationary phase (in a batch culture). They can be more precisely controlled in a continuous culture.
- Secondary metabolism appears to be restricted to some species of plants and microorganisms (and in a few cases to animals). The products of secondary metabolism also appear to be characteristic of the species.
- Secondary metabolites usually have **'bizarre' and unusual chemical structures** and several closely related metabolites may be produced by the same organism in wild-type strains. This latter observation indicates the existence of a variety of alternate and closely-related pathways.

- The ability to produce a particular secondary metabolite, especially in industrially important strains is easily lost. This phenomenon is known as **strain degeneration.**
- Owing to the ease of the loss of the ability to synthesize secondary metabolites, particularly when treated with acridine dyes, exposure to high temperature or other treatments known to induce plasmid loss **secondary metabolite production is believed to be controlled by plasmids** (at least in some cases) rather than by the organism's chromosomes.
- A confirmation of the possible role of plasmids in the control of secondary metabolites is shown in the case of leupetin, in which the loss of the metabolite following irradiation can be reversed by conjugation with a producing parent.
- The factors which trigger secondary metabolism, the inducers, also trigger morphological changes (morphogenesis) in the organism.

Erythromycin tablets: Erythromycin is an example of a secondary metabolite used as an antibiotic and mass produced within industrial microbiology.


Cell Wall Synthesis
Nucleic Acid Synthesis
Folate synthesis
Sulfonamides
Trimethoprim
Beta Lactams
Penicillins
Cephalosporins
Carbapenems
Monobactams
DNA Gyrase
Quinolones
RNA Polymerase
Rifampin
THF A
DHF A
PABA
Vancomycin
Bacitracin
50S
30S
50S subunit
Macrolides
Clindamycin
Linezolid
Chloramphenicol
Streptogramins
Cell Membrane
Polymyxins
30S subunit
Tetracyclines
Aminoglycosides
Protein Synthesis

testbook


Antibiotics
Sulfonamides
Quinolones
Fluro-Quinolones
B-Lactam
Tetracyclins
Macrolides
Aminoglycosides
Sulfamethoxazole
Sulfadiazine
Sulfametho. + Trimethoprim
Others
Nalidixica (1st Gen.)
Enoxacin
Norfluxacin
Ciprofluxacin
Ofluxacin
levo-Fluxacin
Penicillin
Ampicillin
Amoxycillin
Cephalosporins
Others
Doxycyclin
Others
Erythromycin
Azithromycin
Clarithromycin
Clindamycin
Streptomycin
Neomycin
Gentamycin
Kanamycin
Others

Examples of secondary metabolites with importance in industrial microbiology include:

- **Atropine**, derived from various plants, is a secondary metabolite with important use in the clinic. Atropine is a competitive antagonist for acetycholine receptors, specifically those of the muscarinic type, which can be used in the treatment of bradycardia. **Atropine Sulfate Injection is an antimuscarinic agent used to treat bradycardia (low heart rate), reduce salivation and bronchial secretions before surgery, as an antidote for overdose of cholinergic drugs or mushroom poisoning.**
- Antibiotics such as erythromcyin and bacitracin are also considered to be secondary metabolites.
- **Erythromycin**, derived from *Saccharopolyspora erythraea*, is a commonly used antibiotic with a wide antimicrobial spectrum. It is mass produced and commonly administered orally.
- **Bacitracin**, derived from organisms classified under *Bacillus subtilis*, is an antibiotic commonly used a topical drug. **Bacitracin is a topical antibiotic ointment widely used by both medical professionals and the general public to treat minor skin injuries, including cuts, scrapes, and burns. .**

Antibiotic	Produced by	Activity	Chemical Nature
Cephalosporin C	Cephalosporium acremonium	Gram$^+$ and $^-$ bacteria	Peptide
Gentamycin	Micromonospora purpurea	Gram+ bacteria	Aminoglycoside
Griseofulvin	Penicillium griseofulvum	Gram$^+$ and $^-$ bacteria	Spirolactone
Kanamycin	S. kanamyceticus	Gram$^+$ and $^-$ bacteria, and mycobacteria	Aminoglycoside
Neomycins	S. fradiae	Gram$^+$ and $^-$ bacteria	Aminoglycoside
Pimaricin	S. natalensis	Antitumour	Polyene
Penicillin G	P. chrysogenum	Gram+ bacteria	Peptide
Polymixin B	Bacillus polymyxa	Antifungal	Peptide
Streptomycin	S. griceus	Gram$^+$ and $^-$ bacteria, and mycobacteria	Aminoglycoside
Tetracyclines	Streptomyces spp	Gram$^+$ and $^-$ bacteria	Tetracycline

List of antibiotics produced commercially

Table 5.1 Some industrial products resulting from primary metabolism

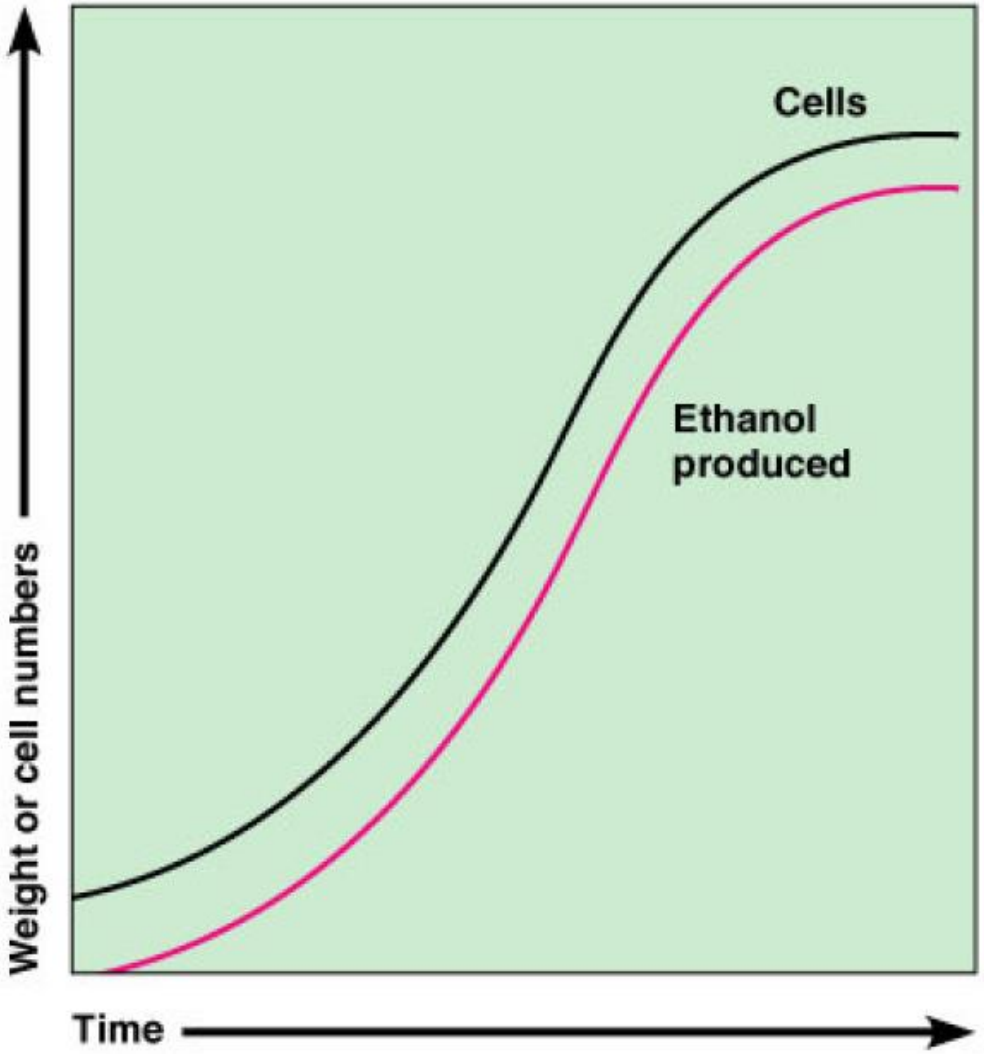
Anabolic Products	*Catabolic Products*
1. Enzymes	1. Ethanol and ethanol-containing products, e.g. wines
2. Amino acids	2. Butanol
3. Vitamins	3. Acetone
4. Polysaccharides	4. Lactic acid
5. Yeast cells	5. Acetic acid (vinegar)
6. Single cell protein	
7. Nucleic acids	
8. Citric acid	

Table 5.2 Some industrial products of microbial secondary metabolism

Product	*Organism*	*Use/Importance*
Antibiotics		
Penicillin	*Penicillium chrysogenum*	Clinical use
Streptomycin	*Streptomyces griseus*	Clinical use
Anti-tumor Agents		
Actinomyin	*Streptomyces antibioticus*	Clinical use
Bleomycin	*Streptomyces verticulus*	Clinical use
Toxins		
Aflatoxin	*Aspergiulus flavous*	Food toxin
Amanitine	*Amanita* sp	Food toxin
Alkaloids		
Ergot alkaloids	*Claviceps purpurea*	Pharmaceutical
Miscellaneous		
Gibberellic acid	*Gibberalla fujikuroi*	Plant growth hormone
Kojic acid	*Aspergillus flavus*	Food flavor
Muscarine	*Clitocybe rivalosa*	Pharmaceutical
Patulin	*Penicillium urticae*	Anti-microbial agent

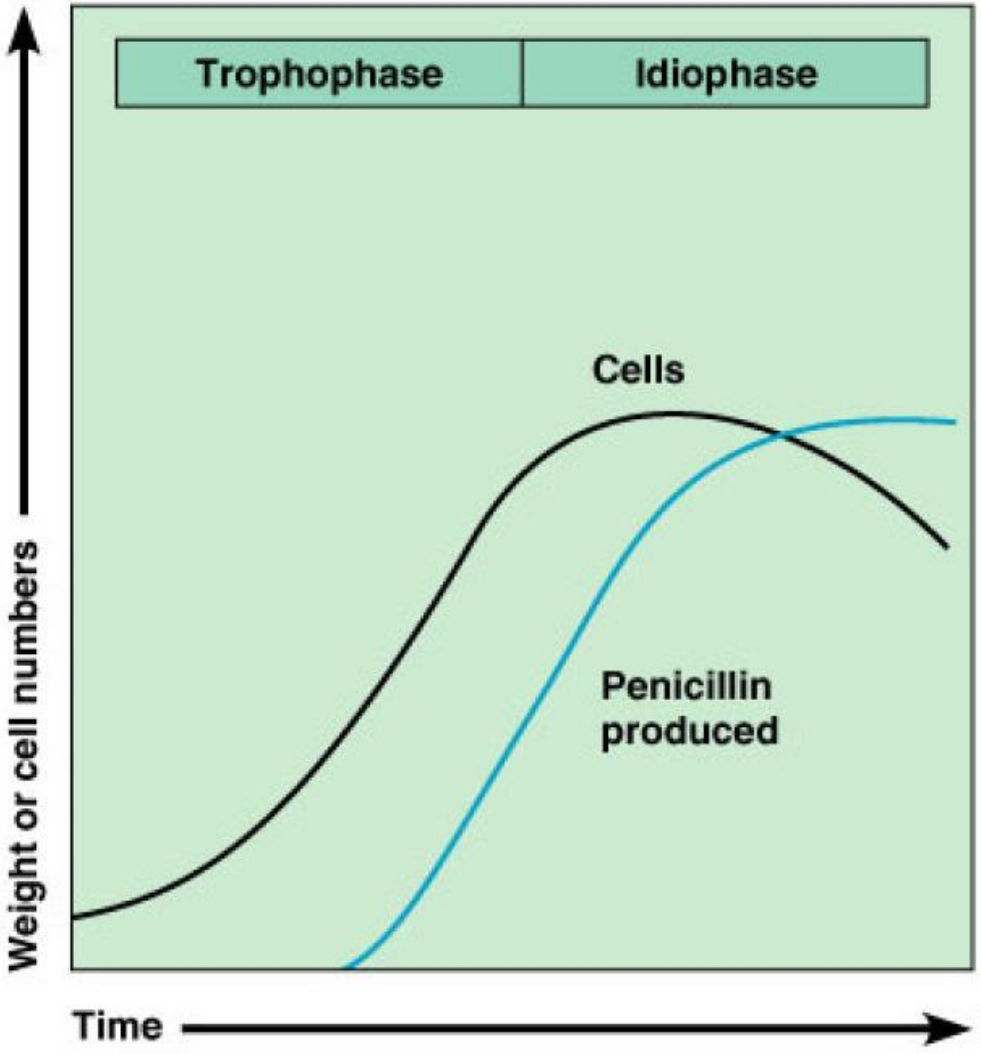
Tropophase and idiophase

- In batch culture, most secondary metabolite processes have a distinct growth phase (trophophase) followed by a production phase (idiophase).
- In other fermentations, the two phases overlap; the timing depends on the nutritional environment presented to the culture, the growth rate, or both.
- A delay in antibiotic production until after trophophase helps the producing organism because the microbe is sometimes sensitive to its own antibiotic during growth.
- Resistance mechanisms that develop in producing microorganisms include enzymatic modification of the antibiotic, alteration of the cellular target of the antibiotic and decreased uptake of the excreted antibiotic.



Primary metabolite

Produced at the log phase of the bacterial growth

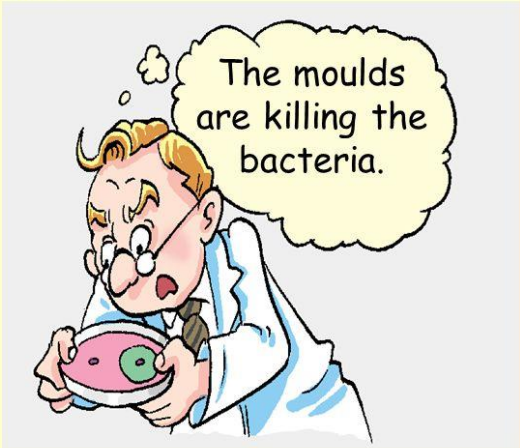


Secondary metabolite

Produced at the idophase of the bacterial growth

One day in 1928...

Dr. Fleming saw some moulds on a dish of bacteria...



In 1945...

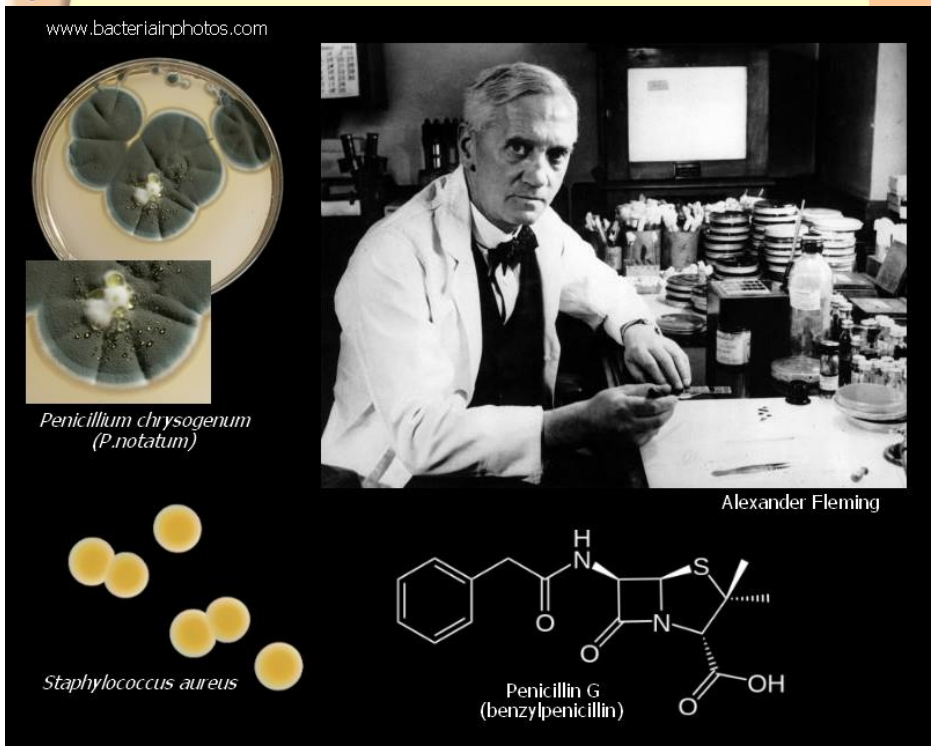
Fleming, Florey and Chain received a Nobel Prize for their work.

What does this story tell us about scientific discoveries?

At that time, bacterial infection killed many people.

Dr. Fleming wanted to find cure for the diseases.

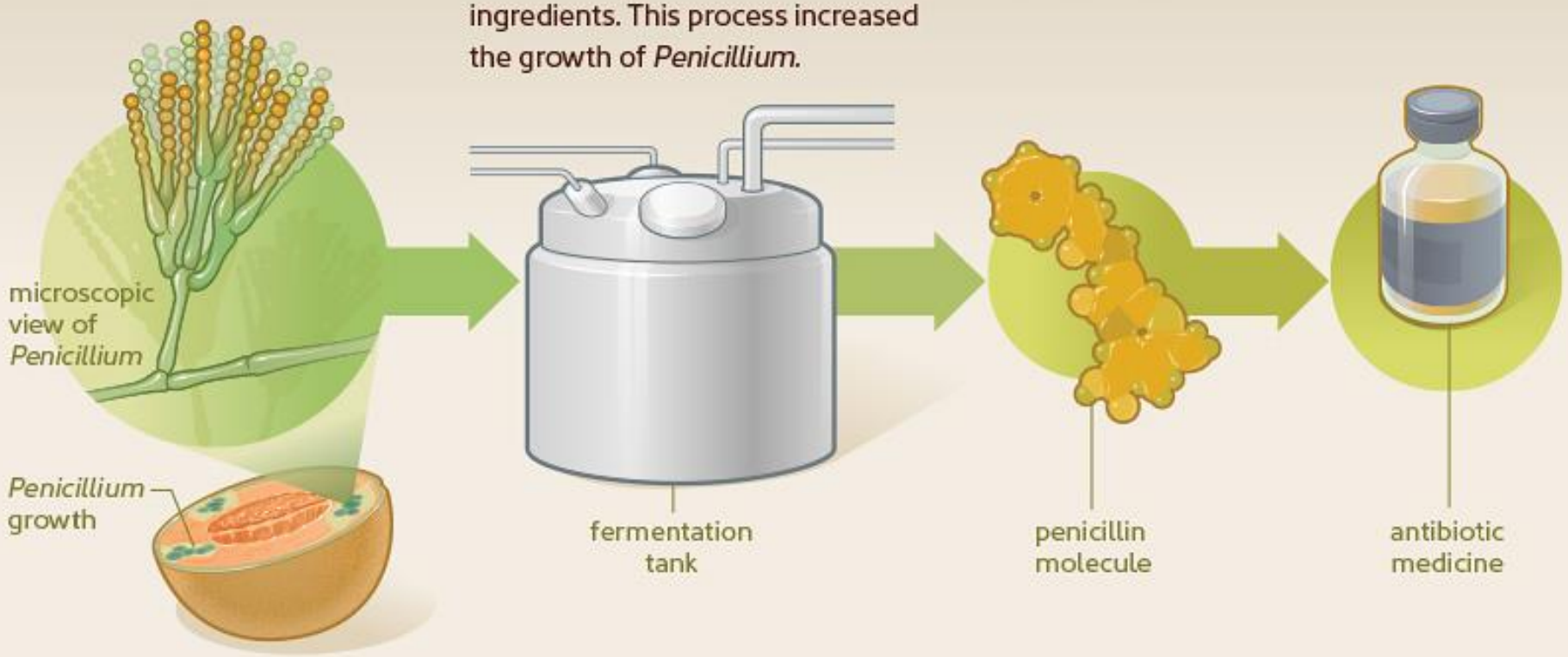
Alexander Fleming (1881-1955)



HOW DID THEY MAKE PENICILLIN?

FOR MANY YEARS, scientists knew that certain molds killed some bacteria. However, researchers needed to understand how to harness this antibacterial microbe and to manufacture enough of the substance before they could make a useful medicine.

1. *Penicillium* mold naturally produces the antibiotic penicillin
2. Scientists learned to grow *Penicillium* mold in deep fermentation tanks by adding a kind of sugar and other ingredients. This process increased the growth of *Penicillium*.
3. Then, scientists separated the penicillin product from the mold.
4. Finally, penicillin is purified for use as an antibiotic medicine.



FERMENTATION PROCESS OF ANTIBIOTICS

MEDIA PREPARATION
medium usually contain its carbon source which is found in corn steep liquor and glucose.

HEAT STERILISATION
Medium is sterilised at high heat and high pressure.

FERMENTATION
Fermentation done in the fed-batch mode. Penicillin is a secondary metabolite of the fungus, so the fed batch mode is ideal as it allows high production of penicillin.
Temperature: 20-24°C
pH: 6.0-6.5.

CULTURE
The seed culture is developed by addition of Penicillium spores into a liquid medium. When it has grown up to an acceptable amount, it will be inoculated into the Fermenter.

BIOMASS REMOVAL
Rotary vacuum filter is commonly used to do this. To maintain the Ph between 6.0-6.5 phosphoric acid is added as the Ph will be high upto 8.5.

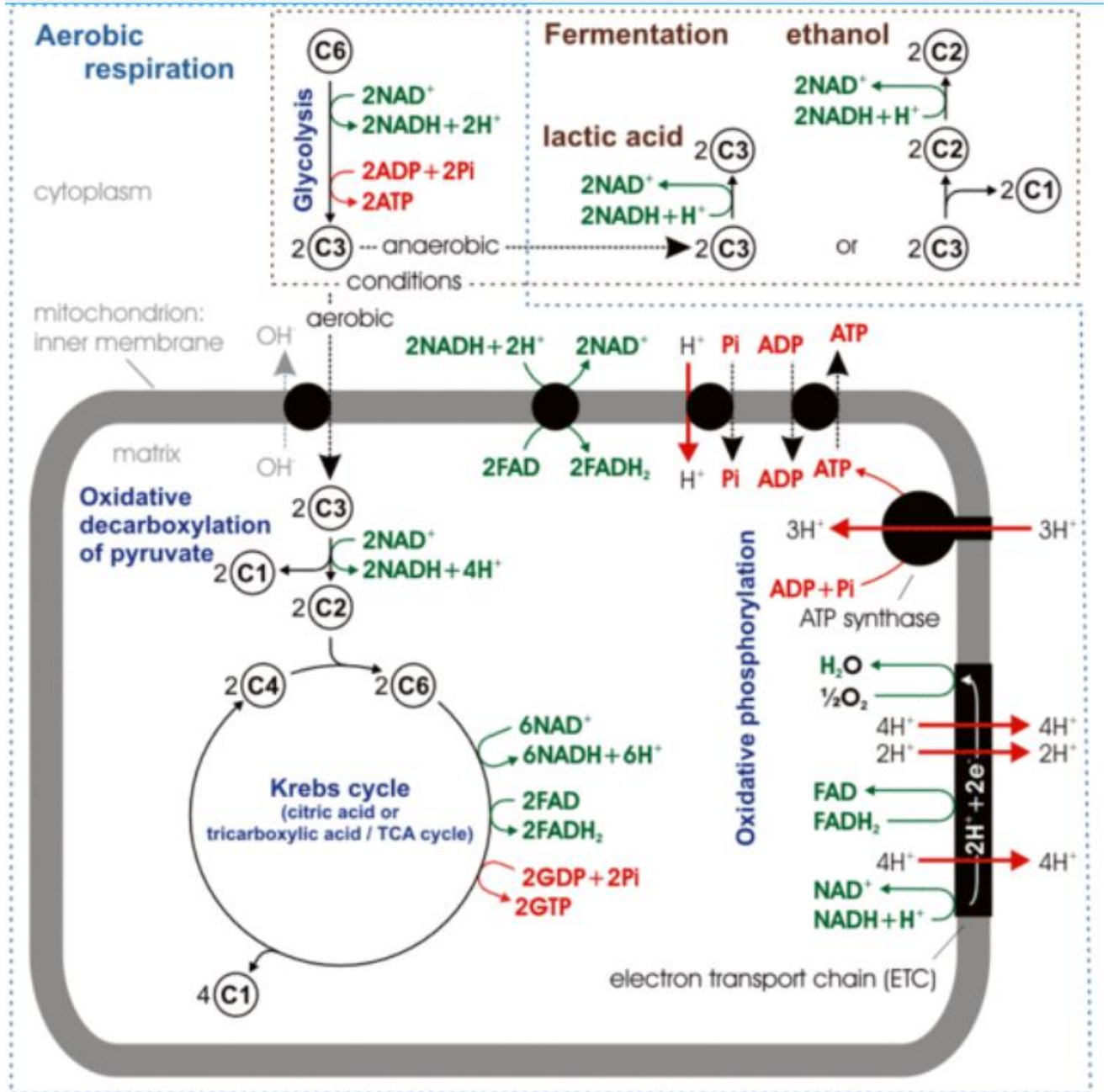
SOLVENT ADDITION
To dissolve the penicillin in the filtrate organic solvent butyl acetate is added.

CENTRIFUGAL EXTRACTION
This is done to separate the solid waste from the liquid solution containing Penicillin.

DRYING
Drying is necessary to completely remove the moisture content from the penicillin salt.

Large-scale fermentations

- Fermentation includes the processes by which energy is extracted from the oxidation of organic compounds.
- The oxidation of organic compounds occurs by utilizing an endogenous electron acceptor to transfer electrons released from nutrients to molecules obtained from the breakdown of these same nutrients.
- There are various types of fermentation which occur at the industrial level such as ethanol fermentation and fermentation processes used to produce food and wine.
- The ability to utilize the fermentation process in anaerobic conditions is critical to organisms which demand ATP production by glycolysis.
- Fermentation can be carried out in aerobic conditions as well, as in the case of yeast cells which prefer fermentation to oxidative phosphorylation.


Aerobic respiration
cytoplasm
C6
Glycolysis
2NAD+
2NADH+2H+
2ADP+2Pi
2ATP
2C3
anaerobic conditions
Fermentation
lactic acid
2C3
2NAD+
2NADH+H+
2C3
ethanol
2C2
2NAD+
2NADH+H+
2C2
2C1
or
2C3
mitochondrion: inner membrane
aerobic
OH-
2NADH+2H+
2NAD+
2FAD
2FADH2
H+ Pi ADP ATP
matrix
Oxidative decarboxylation of pyruvate
2C3
2NAD+
2NADH+4H+
2C1
2C2
2C4
2C6
6NAD+
6NADH+6H+
2FAD
2FADH2
2GDP+2Pi
2GTP
4C1
Krebs cycle
(citric acid or tricarboxylic acid / TCA cycle)
3H+
ADP+Pi
ATP synthase
Oxidative phosphorylation
H2O
½O2
4H+
2H+
FAD
FADH2
2H+ + 2e-
NAD+
NADH+H+
electron transport chain (ETC)

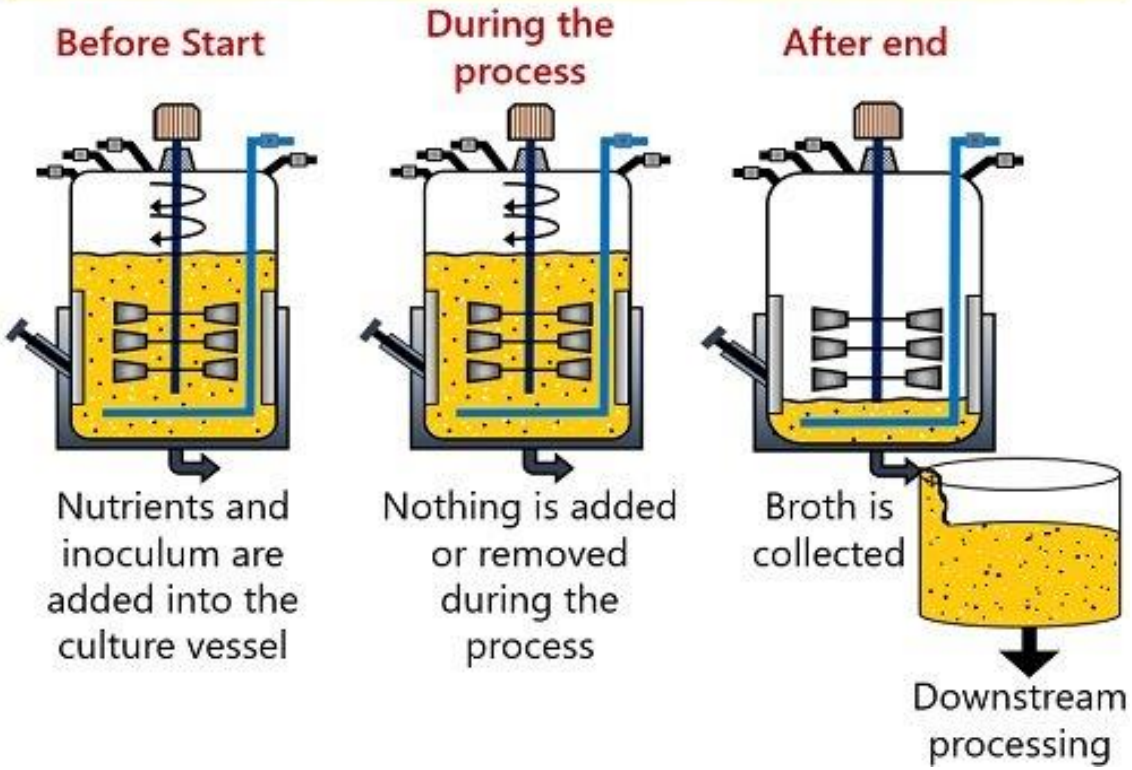
Types of fermentation processes

Industrial fermentation processes may be divided into two main types, with various combinations and modifications. These are:

Batch Fermentation Process

- A tank of fermenter is filled with the prepared mash of raw materials to be fermented. The temperature and pH for microbial fermentation is properly adjusted, and occasionally nutritive supplements are added to the prepared mash. The mash is steam sterilized in a pure culture process. The inoculum of a pure culture is added to the fermenter, from a separate pure culture vessel. Fermentation proceeds, and after the proper time the contents of the fermenter, are taken out for further processing. The fermenter is cleaned and the process is repeated. Thus each fermentation is a discontinuous process divided into batches

Batch Culture Fermentation Process



BATCH FERMENTATION:

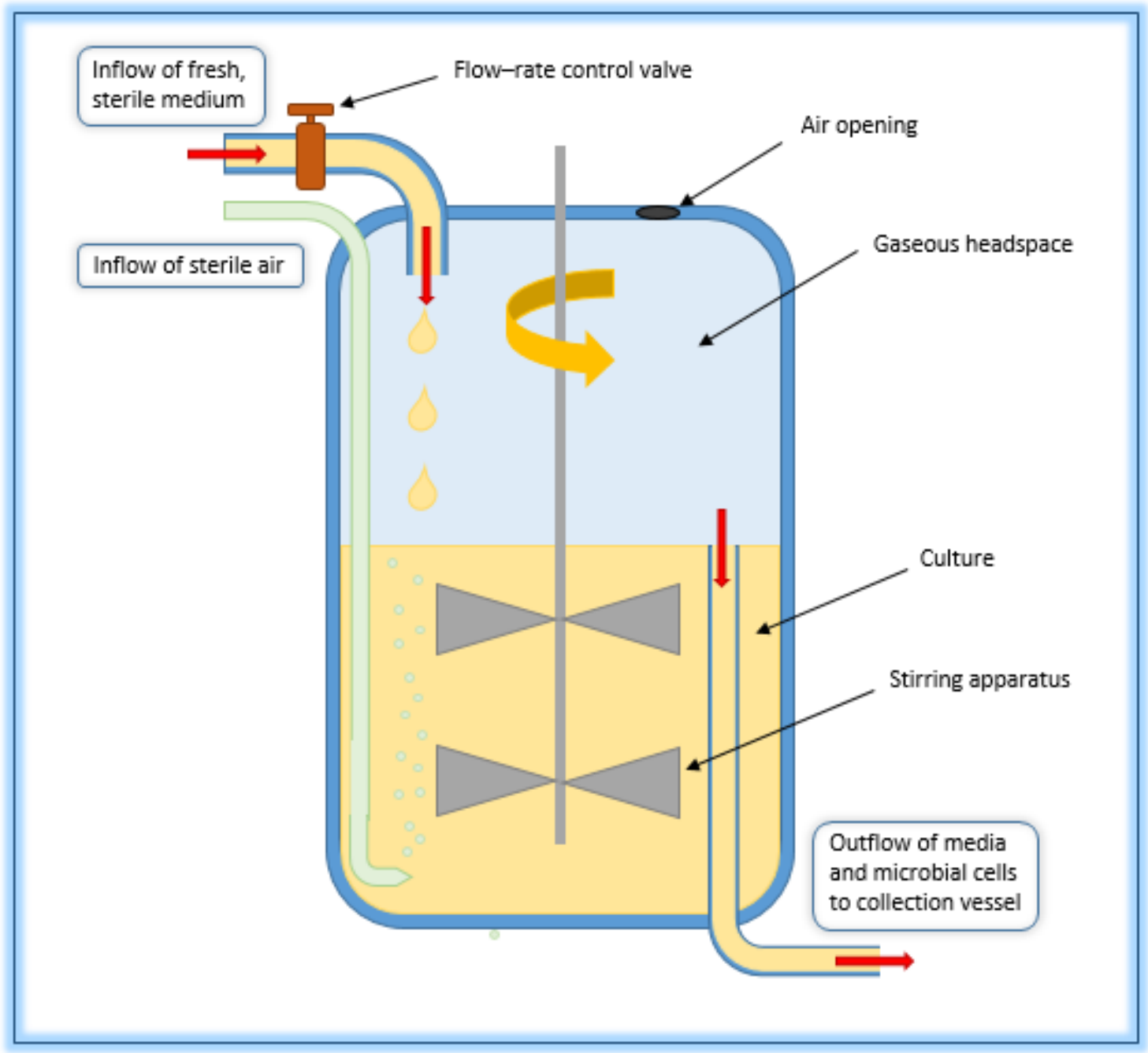
Batch fermentation is a discontinuous process and the fermentor has to be cleaned after each process and a fresh batch started.

It includes the following 5 steps:

1. Medium added
2. Fermentor sterilised
3. Inoculum added
4. Fermentation followed to completion
5. Culture harvested.

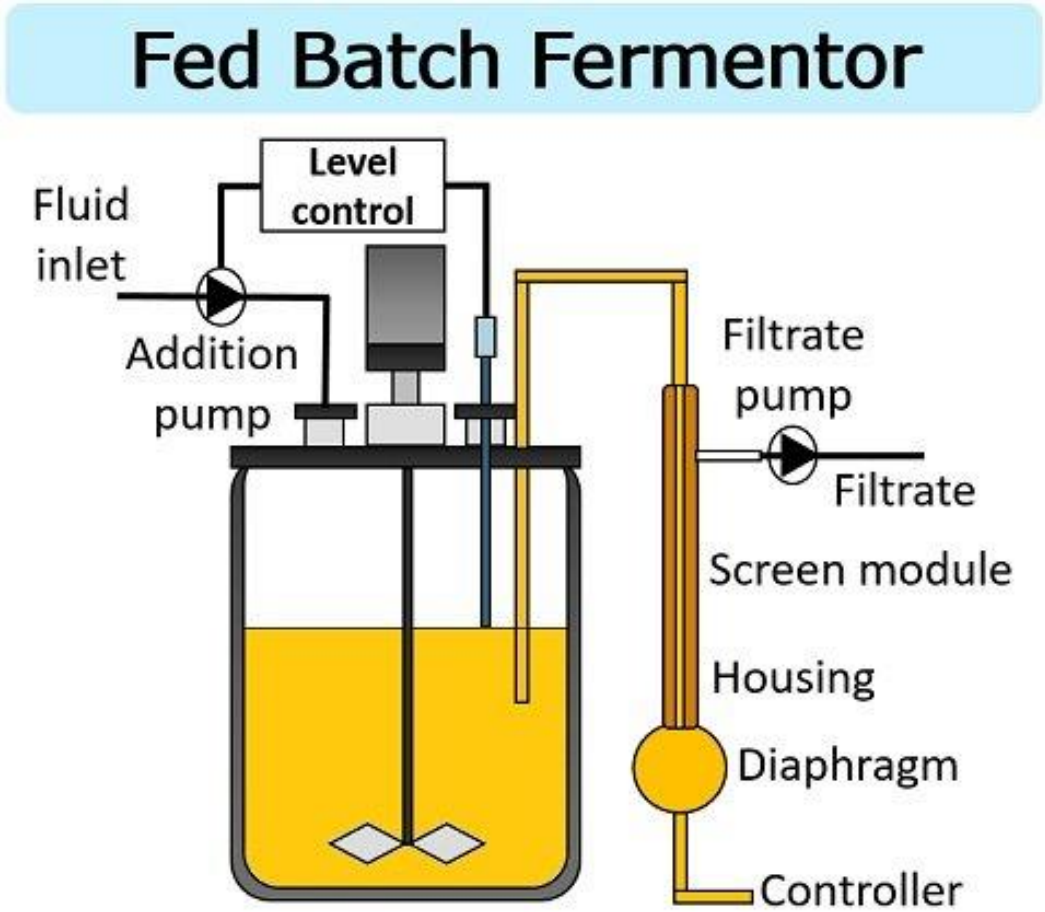
Continuous Fermentation Process

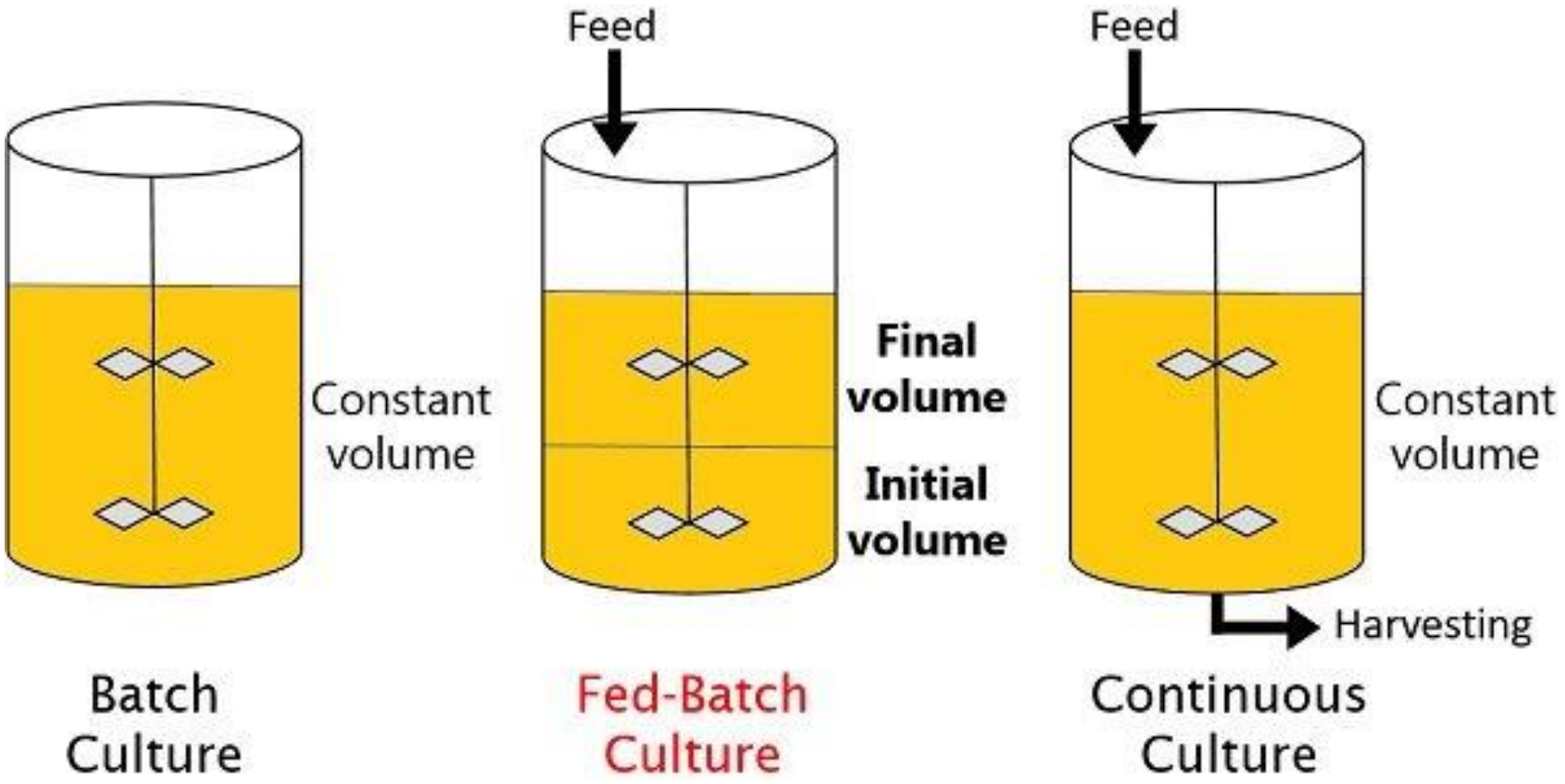
- Growth of microorganisms during batch fermentation confirms to the characteristic growth curve, with a lag phase followed by a logarithmic phase. This, in turn, is terminated by progressive decrements I in the rate of growth until the stationary phase is reached. This is because of limitation of one or more of the essential nutrients. In continuous fermentation, the substrate is added to the fermenter continously at a fixed rate. This maintains the organisms in the logarithmic growth phase. The fermentation products are taken out continuously. The design and arrangements for continuous fermentation, are some what complex.
- Continuous fermentation is **an open operation system with continuous addition and discharge of the solution in the system**. Microorganisms and sterile nutrient solution are added homogeneously to the bioreactor, continuously, while nutrient solution and microorganisms are transformed equivalently in the system.


Inflow of fresh, sterile medium
Flow–rate control valve
Air opening
Inflow of sterile air
Gaseous headspace
Culture
Stirring apparatus
Outflow of media and microbial cells to collection vessel

Fed batch fermentation process:

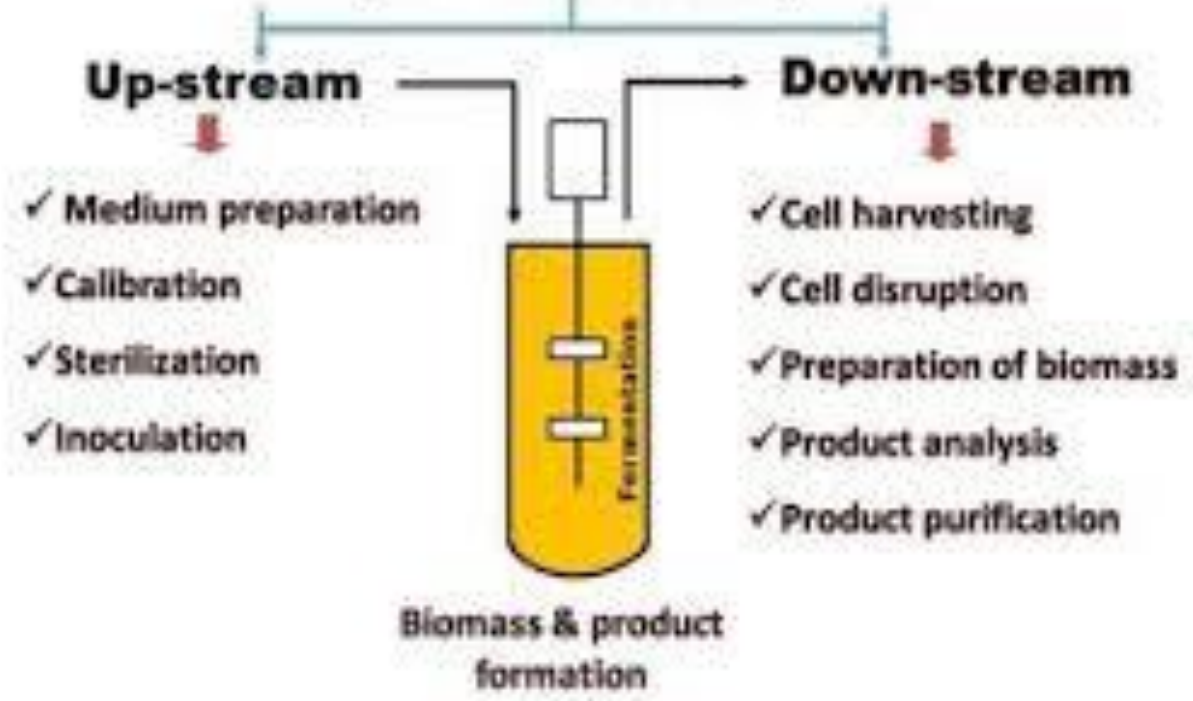
- Fed-batch culture is, in the broadest sense, defined as an operational technique in biotechnological processes where one or more nutrients (substrates) are fed (supplied) to the bioreactor during cultivation and in which the product(s) remain in the bioreactor until the end of the run.





In batch fermentation, all the medium components are placed in the reactor at the start of cultivation. A fed-batch culture is a modification to batch fermentation in which nutrients are systematically added.

Biotechnological process (flow chart)



- **Solid State Fermentation (Semi Solid OR Solid State Methods)** - In this the culture medium is impregnated in a carrier such as bagasse, wheat bran, potato pulp, etc. and the organism is allowed to grow on this. This method allows greater surface area for growth. The production of the desirable substance and the recovery is generally easier and satisfactory.
- **Anaerobic Fermentation -** Basically a fermenter designed to operate under micro aerophilic or anaerobic conditions will be the same as that designed to operate under aerobic conditions, except that arrangements for intense agitation and aeration are unnecessary. Many anaerobic fermentations do, how ever, require mild aeration for the initial growth phase, and sufficient agitation for mixing and maintenance of temperature.
- **Aerobic Fermentation** – A number of industrial processes, although called 'fermentations', are carried on by microorganisms under aerobic conditions. In older aerobic processes it was necessary to furnish a large surface area by exposing fermentation media to air. In modern fermentation processes aerobic conditions are maintained in a closed fermenter with submerged cultures. The contents of the fermenter are agitated with an impeller and aerated by forcing sterilized air.

- **Surface Culture Method** - In this method the organism is allowed to grow on the surface of a liquid medium without agitation. After an appropriate incubation period the culture filtrate is separated from the cell mass and is processed to recover the desirable product. Sometimes the biomass may be reused. Examples of such fermentations are the alcohol production, the beer production and citric acid production. This method is generally time consuming and needs large, area or space.
- **Submerged Culture Method** - In this process, the organism is grown in a liquid medium which is vigorously aerated and agitated in large tanks called fermentors. The fermentor could be either an open tank or a closed tank and may be a batch type or a continuous type and are generally made of non-corrosive type of metal or glass lined or of wood.

Some examples of fermented products:

Ethanol Fermentation

- Ethanol fermentation is used to produce ethanol for use in food, alcoholic beverages, and both fuel and industry.
- The process of ethanol fermentation occurs when sugars are converted into cellular energy.
- The sugars which are most often used include glucose, fructose, and sucrose.

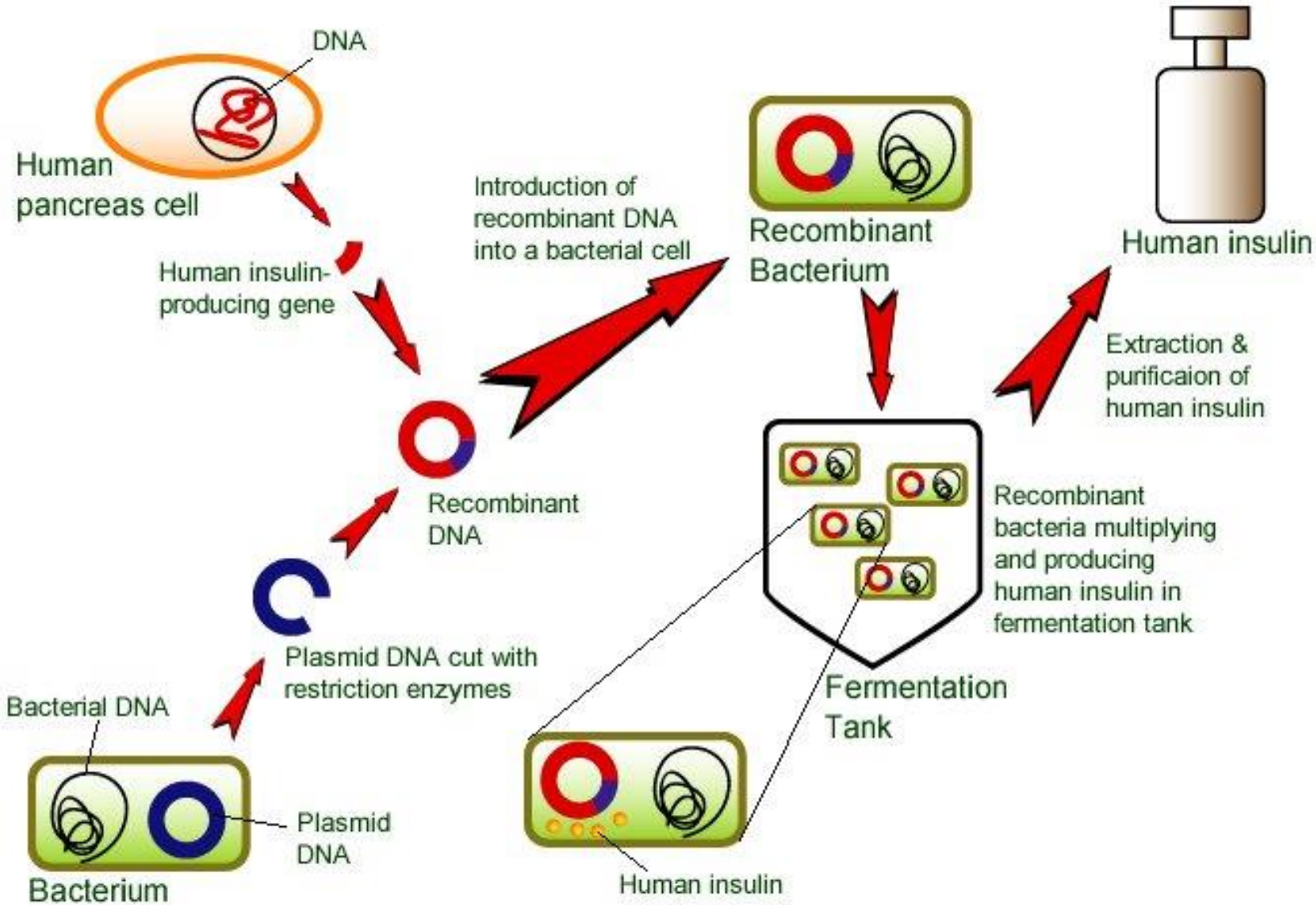
- These sugars are converted into cellular energy and produce both ethanol and carbon dioxide as waste products.
- Yeast is the most commonly used organism to produce ethanol via the fermentation process for beer, wine, and alcoholic drink production.
- As stated previously, despite abundant amounts of oxygen which may be present, yeast prefer to utilize fermentation. Hence, the use of yeast on a large-scale to produce ethanol and carbon dioxide occurs in an anaerobic environment.
- The ethanol which is produced can then be used in **bread production**.
- Yeast will convert the sugars present in the dough to cellular energy and produce both ethanol and carbon dioxide in the process.
- The ethanol will evaporate and the carbon dioxide will expand the dough.
- In regards to alcohol production, yeast will induce fermentation and produce ethanol. Specifically, in wine-making, the yeast will convert the sugars present in the grapes.
- In beer and additional alcohol such as vodka or whiskey, the yeast will convert the sugars produced as a result of the conversion of grain starches to sugar by amylase.
- Additionally, yeast fermentation is utilized to mass produce ethanol which is added to gasoline. The major source of sugar utilized for ethanol production in the US is currently corn; however, crops such as sugarcane or sugar beets can be used as well.

Recombinant Products

Fermentation is also utilized in the mass production of various recombinant products. These recombinant products include numerous pharmaceuticals such as insulin and hepatitis B vaccine.

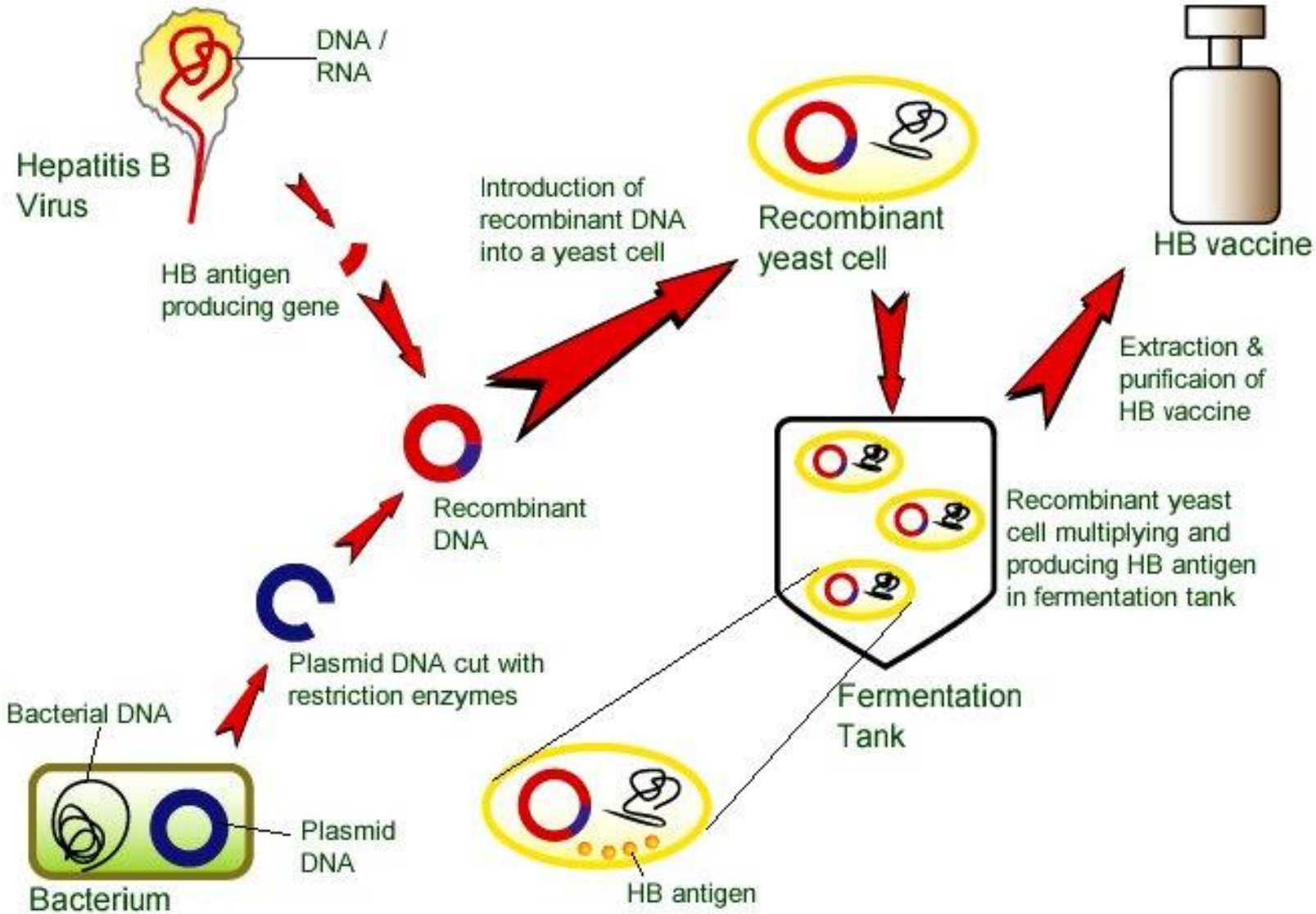
- **Insulin**, produced by the pancreas, serves as a central regulator of carbohydrate and fat metabolism and is responsible for the regulation of glucose levels in the blood.
- Insulin is used medically to treat individuals diagnosed with diabetes mellitus.
- Specifically, individuals with type 1 diabetes are unable to produce insulin and those with type 2 diabetes often develop insulin resistance where the hormone is no longer effective.
- The increase in individuals diagnosed with diabetes mellitus has resulted in an increase in demand for external insulin.
- The mass production of insulin is performed by utilizing both recombinant DNA technology and fermentation processes.
- *E. coli*, *w*hich has been genetically altered to produce proinsulin, is grown to a large amount to produce sufficient amounts in a fermentation broth.
- The proinsulin is then isolated via disruption of the cell and purified.
- There is further enzymatic reactions that occur to then convert the proinsulin to crude insulin which can be further altered for use as a medicinal compound.

Human Insulin Production



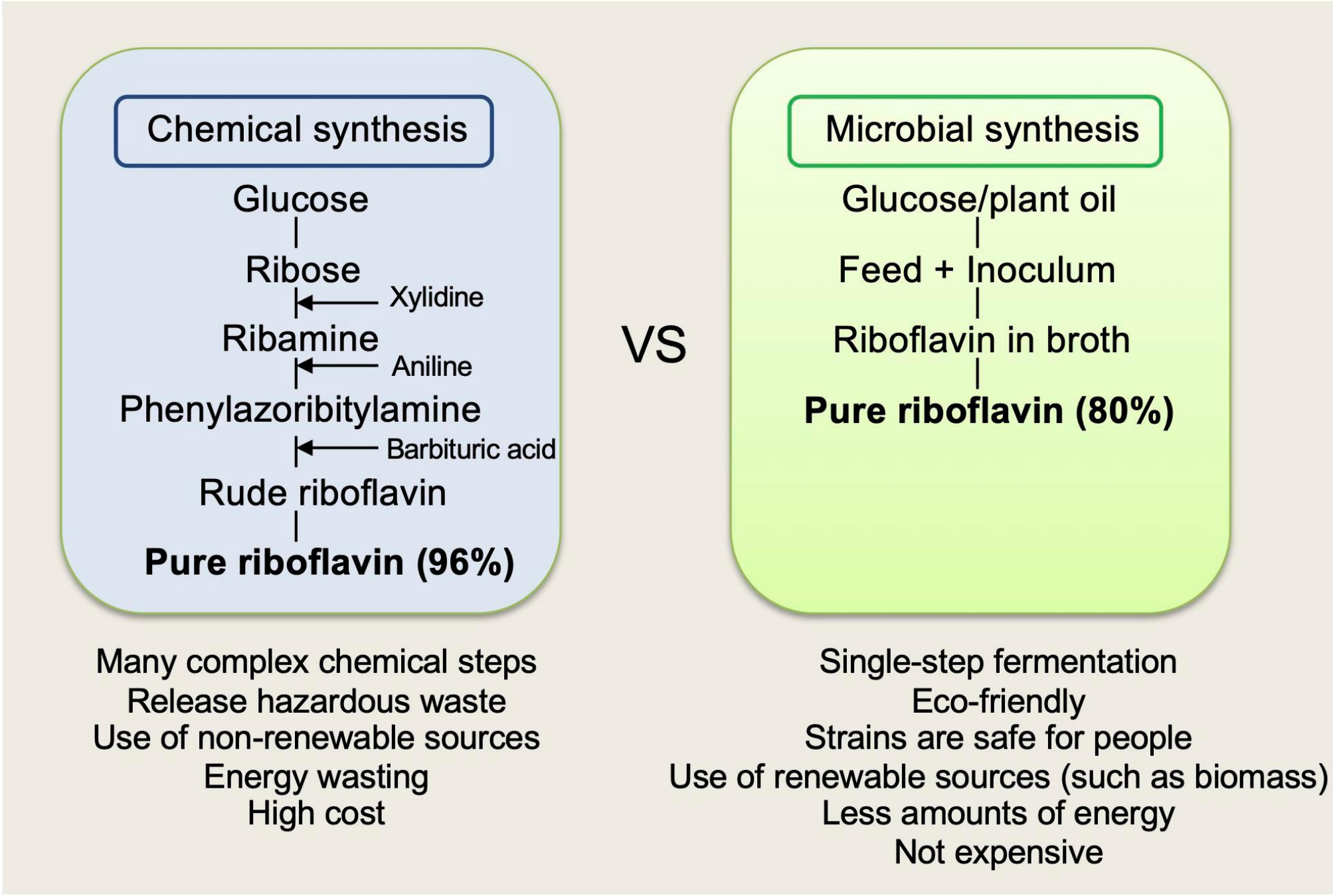
- An additional recombinant product that utilizes the fermentation process to be produced is the **hepatitis B vaccine**.
- The hepatitis B vaccine is developed to specifically target the hepatitis B virus infection.
- The creation of this vaccine utilizes both recombinant DNA technology and fermentation. A gene, HBV, which is specific for hepatitis B virus, is inserted into the genome of the organism yeast.
- The yeast is used to grow the HBV gene in large amounts and then harvested and purified. The process of fermentation is utilized to grow the yeast, thus promoting the production of large amounts of the HBV protein which was genetically added to the genome.

Production of Recombinant HB Vaccine



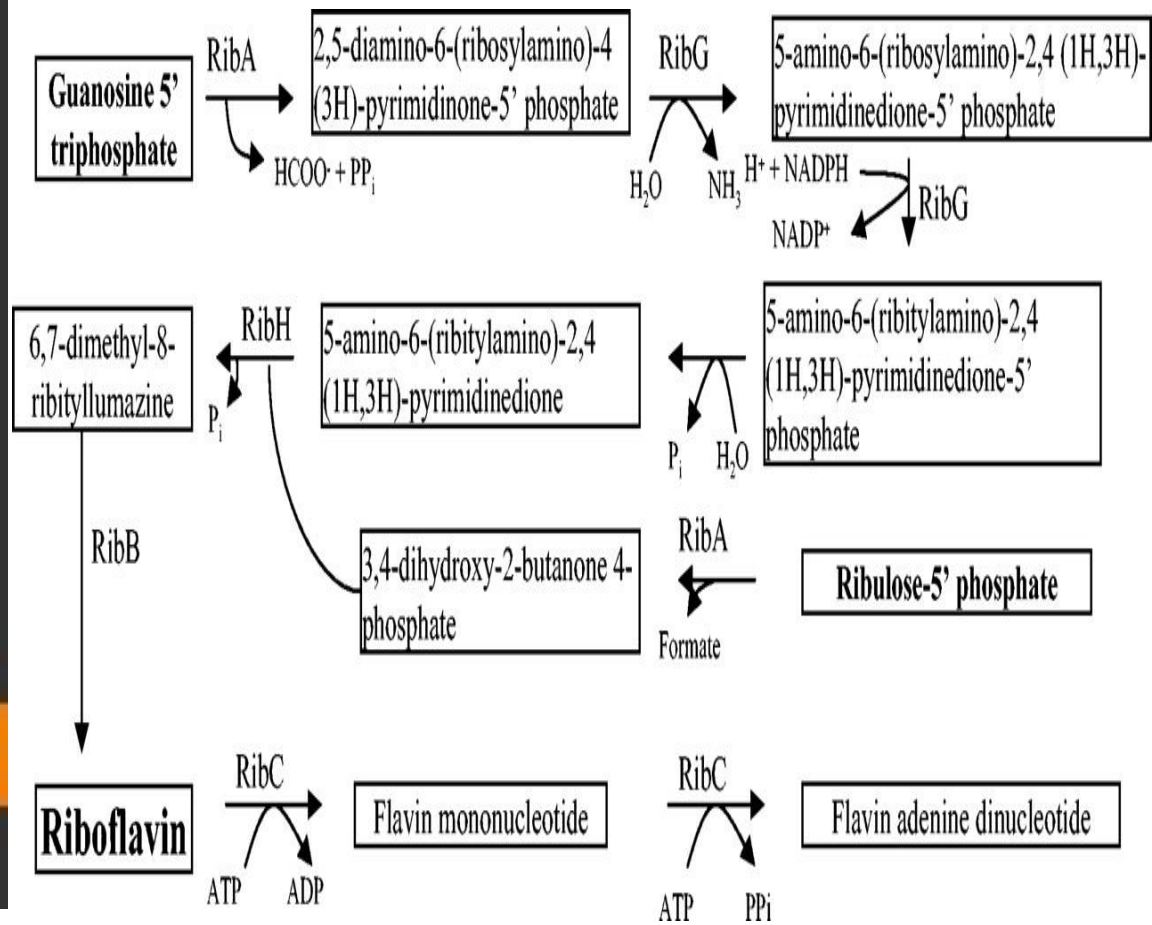
Vitamins

- Riboflavin (vitamin B2) overproducers include two yeast-like molds, *Eremothecium ashbyii* and *Ashbya gossypii*, which synthesize riboflavin in concentrations greater than 20 g.
- New processes using *Candida* sp. or recombinant *Bacillus subtilis* strains that produce up to 30 g riboflavin have been developed in recent years.
- Vitamin B12 is produced on an industrial scale by *Propionibacterium shermanii* or *Pseudomonas denitrificans*.
- The key to the fermentation is to avoid feedback repression by vitamin B12.
- The early stage of the *P. shermanii* fermentation is conducted under anaerobic conditions in the absence of the precursor 5,6-dimethylbenzimidazole.
- These conditions prevent vitamin B12 synthesis and allow for the accumulation of the intermediate, cobinamide. The culture is then aerated and dimethylbenzimidazole is added, converting cobinamide to the vitamin.
- In the *P. denitrificans* fermentation, the entire process is carried out under low oxygen. A high level of oxygen results in an oxidizing intracellular environment that represses the formation of the early enzymes in the pathway.
- Production of vitamin B12 has reached levels of 150 mg l21 and a world market value of US$71 million.

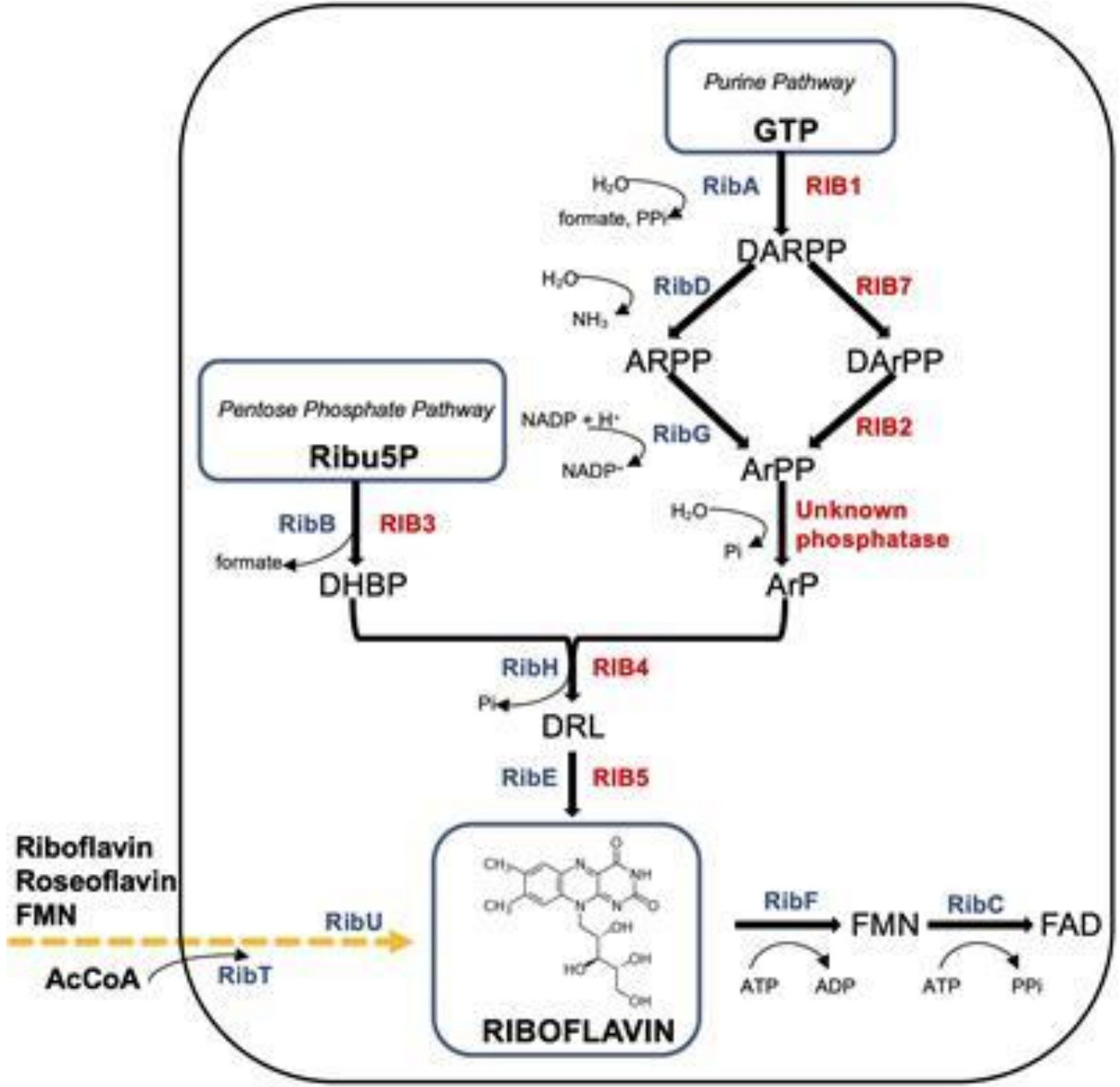

Chemical synthesis
Glucose
Ribose
Xylidine
Ribamine
Aniline
Phenylazoribitylamine
Barbituric acid
Rude riboflavin
Pure riboflavin (96%)
VS
Microbial synthesis
Glucose/plant oil
Feed + Inoculum
Riboflavin in broth
Pure riboflavin (80%)
Many complex chemical steps
Release hazardous waste
Use of non-renewable sources
Energy wasting
High cost
Single-step fermentation
Eco-friendly
Strains are safe for people
Use of renewable sources (such as biomass)
Less amounts of energy
Not expensive

Production Process

- In this case study, a batch process using E. Ashbyii with a capacity of around 1000 tons/year is analyzed.
- Upstream processing consists of preparation of medium and associated continuous counter-current sterilization.
- Feed components are: 70% glucose syrup, yeast and malt extract, sunflower oil, sulfuric acid, and concentrated salt solution at room temperature.
- Fermentation is operated batch-wise with 10% inoculum ratios.
- Downstream processing starts with harvesting followed by crystallization, centrifugation (decanter), and final drying (spray dryer).
- The requested purity of riboflavin is 70%. The residual 30% consists of salts and biomass. The product is obtained as dry powder or as granulate.



Riboflavin synthesis starts from GTP and ribulose-5-phosphate and proceeds through pyrimidine and pteridine intermediates. Flavin nucleotides are synthesized in two consecutive reactions from riboflavin.


Purine Pathway
GTP
RibA
RIB1
H2O
formate, PPi
DARPP
RibD
RIB7
H2O
NH3
ARPP
DArPP
Pentose Phosphate Pathway
Ribu5P
NADP + H+
RibG
NADP+
RIB2
ArPP
H2O
Unknown phosphatase
Pi
RibB
RIB3
formate
DHBP
ArP
RibH
RIB4
Pi
DRL
RibE
RIB5
Riboflavin
Roseoflavin
FMN
RibU
AcCoA
RibT
RIBOFLAVIN
RibF
FMN
RibC
FAD
ATP
ADP
ATP
PPi

Non-antibiotic agents

- In nature, secondary metabolites are important to the organisms that produce them, functioning as: (1) sex hormones; (2) ionophores; (3) competitive weapons against other bacteria, fungi, amoebae, insects and plants; (4) agents of symbiosis; and (5) effectors of differentiation.
- Many microbial products with important pharmacological activities were discovered by screening for inhibitors using simple enzymatic assays. **One huge success has been the statins, including lovastatin (also known as mevinolin) and pravastatin: fungal products that are used as cholesterol-lowering agents in humans and animals. In its hydroxy acid form, lovastatin is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl- coenzyme A reductase from liver.**
- Other well known enzyme inhibitors include: **clavulanic acid, a penicillinase-inhibitor** that protects penicillin from inactivation by resistant pathogens; and **acarbose, a natural inhibitor of intestinal glucosidase,** which is produced by an actinomycete of the genus *Actinoplanes.*
- Acarbose decreases hyperglycemia and triglyceride synthesis in adipose tissue, the liver and the intestinal wall of patients suffering from diabetes, obesity and type IV hyperlipidemia.

Also in commercial or near-commercial use are:

- biopesticides, including biofungicides (e.g. kasugamycin, polyoxins), bioinsecticides (nikkomycin, spinosyns), bioherbicides (bialaphos), antihelminthics (avermectin), coccidiostats,
- ruminant-growth promoters (monensin, lasalocid, salinomycin),
- plant-growth regulators (gibberellins),
- immunosuppressants for organ transplants (cyclosporin A, FK-506, rapamycin),
- anabolic agents in farm animals (zearelanone),
- uterocontractants (ergot alkaloids) and
- antitumor agents (doxorubicin, daunorubicin, mitomycin, bleomycin).