Riboflavin (Vit B2) Production

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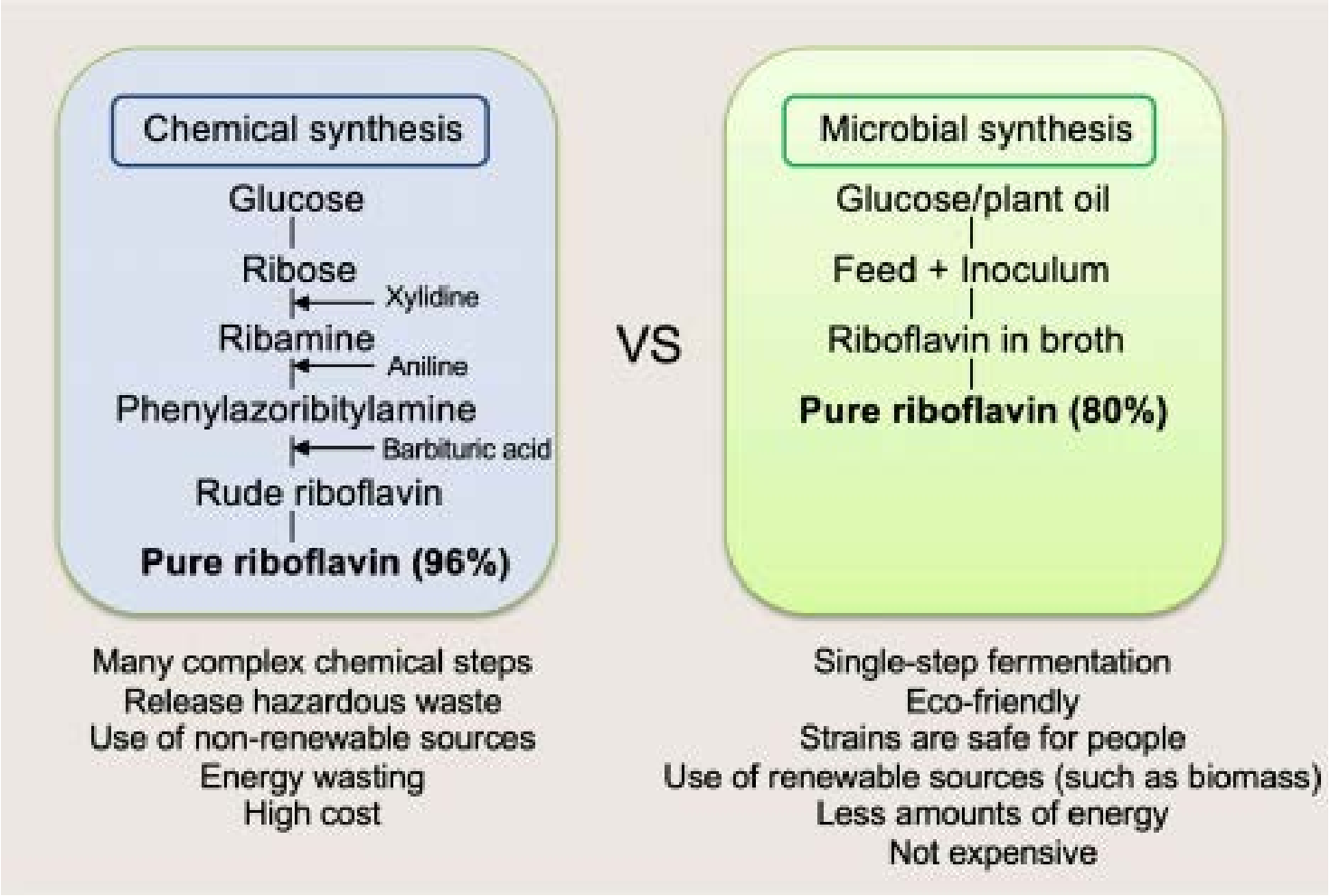
Riboflavin

- Riboflavin (RF, commonly known as vitamin B2) is the central source of all biologically important flavins.
- RF was discovered in 1879 as a yellow pigment from milk and its chemical structure was deciphered in the 1930s.
- Its derivatives flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are indispensible as active groups in the majority of flavoproteins/flavo-coenzymes.
- Flavins are broadly distributed in tissues, but are rarely present as free RF. Instead, most are bound to flavoproteins, mainly as FAD and lesser amounts as FMN.
- Humans and livestock must obtain RF from the diet because they have lost the ability of its de-novo synthesis.
- Riboflavin exerts its biochemical functions through the coenzymes namely flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN).
- RF deficiency may lead to increased risk of cardiovascular disease, impairment of iron metabolism and night blindness.
- Flavins are used for the treatment of ariboflavinosis, a condition marked by lesions in the corners of the mouth, on the lips, and around the nose and eyes, or as general health supplements in the case of malnutrition.

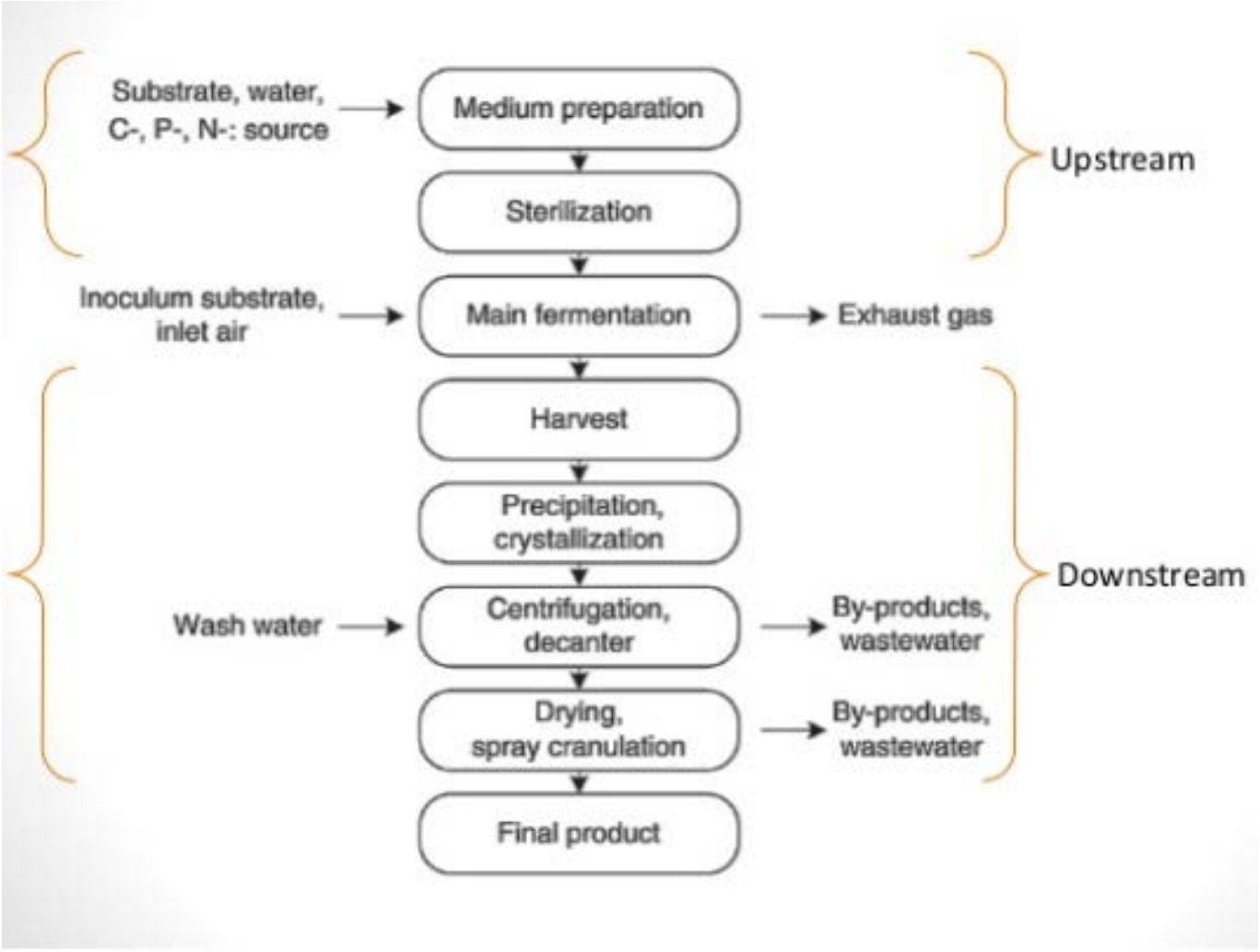
Riboflavin Production

- There are three processes employed for the large scale production of riboflavin. The worldwide requirement of riboflavin is estimated to be around 2,500 tones per year.
- **1. Biotransformation:**
- About 50% of the world's requirement of riboflavin is produced by biotransformation, followed by chemical synthesis. For this purpose, glucose is first converted to D-ribose by mutant strains of *Bacillus pumilus*. The D-ribose so produced is converted to riboflavin by chemical reactions.
- **2. Chemical synthesis:**
- Approximately 20% of the world's riboflavin is produced by direct chemical synthesis.
- Chemical synthesis of RF essentially consisted of six to eight chemical steps starting from d-glucose or D-ribose.
- **3. Fermentation:**
- At least one third of world's riboflavin requirements are met by direct fermentation processes.
- 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) (Now not in use, because it was unproftable due to the low stability of the strain)
  - A genetically engineered strain of *Bacillus subtilis* (DSM, Germany)
- The overproduction of riboflavin in these organisms takes place mainly due to the constitutive nature of the riboflavin synthesizing enzymes.

Chemical versus microbial riboflavin synthesis



Microbial Production of Riboflavin



Ashbya gossypii & Fermentation Process

- Commercial production of riboflavin is predominantly carried out by direct fermentation using the ascomycetes.
- The two plant pathogens namely *Ashbya gossypii* and *Eremothecium ashbyii* are most commonly employed due to high yield.
- Among these two organisms, *A. gossypii* is preferred as it is more stable with a high producing capacity of riboflavin.
- High yielding strains of *A. gossypii* have been developed by genetic manipulations. Such strains can yield as high as 15 g/l riboflavin.

Production process of riboflavin

- Industrial production of riboflavin is mostly carried out with the organism, *Ashbya gossypii* by using simple sugars such as glucose and corn steep liquor.
- Glucose can be replaced by sucrose or maltose for the supply of carbon source.
- In recent years, lipids such as corn oil, when added to the medium for energy purpose, have a profound influence on riboflavin production.
- Further, supplementation of the medium with yeast extract, peptones, glycine, inositol, purines (not pyrimidine's) also increase the yield of riboflavin.

Fermentation conditions

- The initial pH of the culture medium is adjusted to around 6-7.5.
- The fermentation is conducted at temperature 26-28°C with an aeration rate 0.3 vvm.
- The process is carried out for about 5-7 days by submerged aerated fermentation.
- Riboflavin fermentation by *Eremothecium ashbyii* is comparable to that described above for *Ashbya gossypii*.
- *Candida* sp. can also produce riboflavin, but this fermentation process is extremely sensitive to the presence of iron.
- Consequently, iron or steel equipment cannot be used. Such equipment have to be lined with plastic material.

Phases of Fermentation

- Some studies have been carried out to understand the process of fermentation of riboflavin particularly by ascomycetes.
- It is now accepted that the fermentation occurs through three phases.

Phase I:

- This phase is characterized by rapid growth of the organism utilizing glucose.
- As pyruvic acid accumulates, pH becomes acidic.
- The growth of the organism stops as glucose gets exhausted.
- In phase I, there is no production of riboflavin.

Phase II:

- Sporulation occurs in this phase, and pyruvate concentration decreases.
- Simultaneously, there is an accumulation of ammonia (due to enhanced deaminase activity) which makes the medium alkaline.
- Phase II is characterized by a maximal production of riboflavin.
- But this is mostly in the form of FAD and a small portion of it as FMN.

Phase III:

- In this last phase, cells get disrupted by a process of autolysis.
- This allows release of FAD, FMN and free riboflavin into the medium.

Recovery

- Riboflavin is found in fermentation broth and in a bound form to the cells.
- The latter can be released by heat treatment i.e. 120°C for about 1 hour.
- The cells can be discarded after filtration or centrifugation.
- The filtrate can be further purified and dried, as per the requirements.

Bacillus subtilis Strain & Fermentation Process

- The first step to increase the production of riboflavin was through the selection of mutants resistant to purine such as 8-azaguanine, methionine sulfoxide.
- Other strategies such as genetic engineering of the strain was adopted.
- The fermentation process is also conducted in a carbon-source-limited fed-batch.
- Molasses and thick juices are used as a carbon source.
- For a nitrogen source corn steep liquor and yeast extract can be utilized.
- The *B. subtilis* fermentation process is conducted at 39 to 40° and last up to 70h.
- The influence of agitation speed and oxygen supply is important on a riboflavin fed-batch production process.
- Optimal cell growth and riboflavin production was identified when the agitation speed during the first process phase was lower (600rpm) compared to the agitation speed in the later phase of the fermentative process (900rpm).
- This process effectively produces riboboflavin from glucose in fed-batch operation.
- Recombinant *B.subtilus* can yield up to 16g/L riboflavin in 48hours.
- Riboflavin is sparingly soluble and forms crystals in the fermentation broth.
- *Bacillus subtilis* is much small than the produced riboflavin particles, making downstream purification easier.

Other carbon sources and microorganisms for riboflavin production

- A pure grade of riboflavin can be prepared by using *Saccharomyces* sp., utilizing acetate as sole carbon source.
- Methanol-utilizing organism *Hansenula polymorpha* was found to produce riboflavin.
- The other carbon sources used with limited success for riboflavin production are aliphatic hydrocarbons (organism *Pichia guilliermoudii*) and n-hexadecane (organisms — *Pichia miso*).